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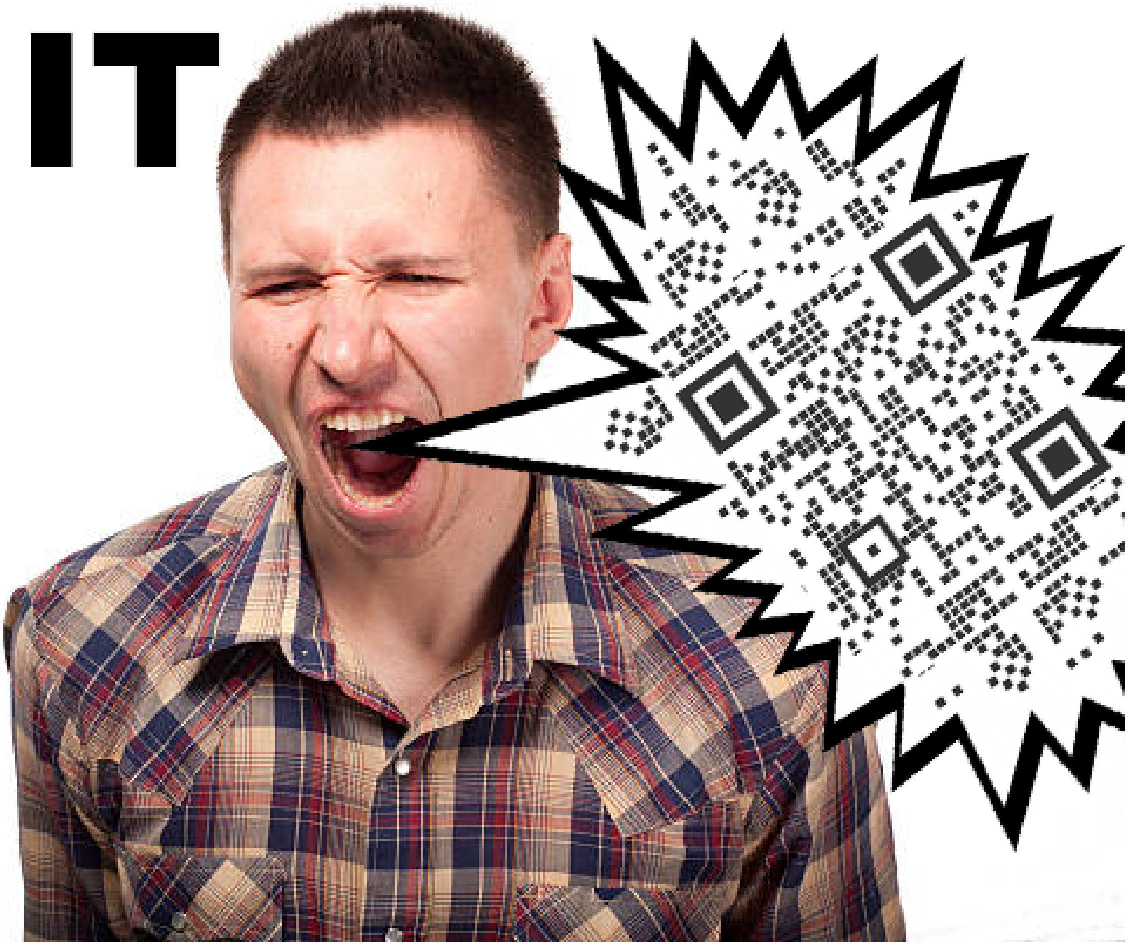

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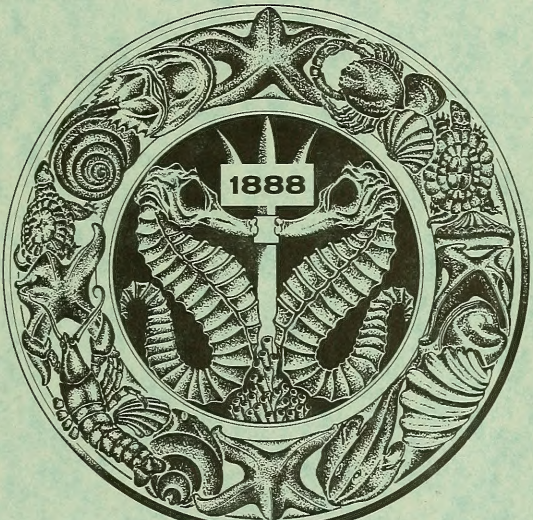

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Convergence in the Feeding Apparatuses of Lophophorates and Pterobranch Hemichordates Revealed by 18S rDNA: An Interpretation

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Homology of the feeding apparatus of pterobranch hemichordates and the lophophore of bryozoans, brachiopods, and phoronids has been postulated but never rigorously tested. I employ Patterson's (1, 2) three criteria of conjunction, similarity, and congruence to test this putative homology. Although the conjunction and similarity criteria are satisfied, congruence is not. The congruence test is based on a phylogeny derived from 18S rDNA sequence data which show that pterobranchs and lophophorates are in different metazoan subkingdoms and are not closely related. This finding indicates that a lophophore-like apparatus has evolved at least twice in metazoans even though the gross morphology, feeding mechanics, ultrastructure, and ciliary patterns of these organisms are very similar. The high degree of morphological convergence presumably results from similar selective regimes acting on these taxa. These findings indicate that major clades of organisms can evolve in a corresponding fashion despite independent origins.

The lophophorates (consisting of the phoronids, brachiopods, and bryozoans) and the pterobranch hemichordates are sessile suspension-feeding marine organisms that use ciliated tentacles to capture algae. These tentacles are unique among metazoans because they have similar ciliation patterns, are invaded by the mesocoelomic cavity, and surround the mouth but not the anus. Several workers have postulated that the lophophores of brachiopods, bryozoans, and phoronids are homologous to the tentaculate arms of pterobranch hemichordates (3–8), leading to phylogenetic hypotheses that support the notion of homology among these structures (8, 9). However, most traditional phylogenies show these taxa to be unrelated (3, 10), suggesting convergence.

In this research, a phylogeny based on 18S rDNA sequence data is used to test the putative homology of the feeding apparatuses found in the Lophophorata and Pterobranchia. Patterson's (1, 2) three criteria of conjunction, similarity, and congruence constitute the framework for the test. For these purposes, a homologous structure is defined as a feature in two or more groups of organisms that was derived from a single common feature present in a shared ancestor. That is, Patterson's notion of homology, which equates homology and synapomorphy, is adopted here. To be considered homologous, the morphological feature or pattern in question must satisfy all three of Patterson's criteria (described in turn).

If the structures in question are "anatomical singularities," then the conjunction criterion has been met. The main utility of this criterion is that it distinguishes homology by descent from homology due to serial repetition (see 1, 2). An examination of the tentacles in the lophophorate and pterobranch feeding apparatus provides clear evidence that serial repetition has occurred: (i) closely related species have different numbers of tentacles, (ii) the internal structure of the tentacles within an organism is the same, and (iii) the tentacles develop in a progressive series (3, 11; pers. obs.).

The issue, however, is whether the feeding apparatus as a whole has been serially repeated. Although phoronids, bryozoans, brachiopods, and rhabdopleurid pterobranchs each have a single bilateral tentacular feeding structure that surrounds the mouth but not the anus, the cephalodiscid pterobranchs have multiple arms bearing tentacles (6, 12). Given that the males of *Cephalodiscus sibogae* and all *Rhabdopleura* species have a single pair of arms (there are only two pterobranch genera; *Atubaria* is most likely a form of *Cephalodiscus*), the ancestral condition

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for pterobranchs is assumed, by parsimony, to be a single pair of arms because a single evolutionary event can account for the multiple arms in *Cephalodiscus.* Therefore, the feeding apparatus in lophophorates and pterobranchs satisfies the conjunction test.

Similarity is perhaps the most obvious criterion for homology. The similarity between the feeding apparatus of pterobranchs and the lophophore of bryozoans, brachiopods, and phoronids has long been known; the earliest reports on the pterobranchs considered them either related to, or included in, the Bryozoa (13–15). In both the pterobranchs and the lophophorates, the feeding apparatus is a ciliated tentacular structure that is invaded by the mesocoelomic cavity and surrounds the mouth but not the anus. (Although in some pterobranchs the mesosome completely surrounds the mouth, tentacles are not present between the mouth and the region in which the cephalic shield attaches.) This arrangement is fundamentally different from that of other suspension-feeding organisms such as entoprocts (in which both the mouth and anus are surrounded by tentacles) or some polychaetes (*e.g.,* serpulids, in which several segments of the organism are devoted to the tentacular apparatus). Except in bryozoans and some brachiopods, blood vessels are usually found in close association with the tentacular coelom (3, 7, 16, 17).

Moreover, the resemblance between these structures extends to ultrastructure and functional morphology (7, 17 and refs. therein, 18). In all of these taxa, the arrangement of the cilia on the tentacles is identical. Three ciliated bands (lateral, frontolateral, and frontal) are present along the length of the tentacle. The lateral cilia generate a current that draws water toward the frontal surface of the arm between and perpendicular to the tentacles (see fig. 2 in 18). In the suspension-feeding entoprocts, the direction of water flow is reversed (3). As the water passes through the suspension-feeding apparatus of lophophorates and pterobranchs, food particles are captured with an upstream particle-collection mechanism by a local reversal in beat of the lateral cilia (8, 17, 18). Once a particle has been captured, frontolateral and frontal ciliary bands transport it down the length of the tentacle without the aid of mucus. Other suspension-feeding organisms (*e.g.,* serpulid polychaetes and crinoids) use mucus during particle transport. These taxa all reject particles by using the frontolateral and frontal cilia to transport particles distally until they fall off the tentacle (in addition to other rejection methods). Unfortunately, information on the development of pterobranch arms and tentacles is so limited that a meaningful comparison to lophophorate tentacular development cannot be made. In essence, however, the similarity criterion supports the putative homology among these structures.

Although the conjunction and the similarity criteria of homology are often met, congruence is the most discriminating criterion of the three. Congruence refers to correspondence of the homology in question to other putative or known homologies. In the case of lophophorate and pterobranch feeding morphology, an *a priori* assessment of which morphological features are evolving independently cannot be accurately made. The sessile nature of lophophorates and pterobranchs has undoubtedly caused evolutionary influences on their reproduction, feeding, and morphology. The question is which features have evolved relatively independently. For example, two potential phylogenetic characters, a U-shaped gut and a circumoral ciliated ring of tentacles, might both be consequences of a single feature (a sessile existence) because they are found in several unrelated sessile organisms. Lophophorates, pterobranchs, and urochordates have U-shaped guts. Sessile polychaetes, lophophorates, pterobranchs, and entoprocts all have ciliated circumoral tentacles. Thus, in this case, we cannot accurately test the homology of the feeding apparatus by using a phylogeny based on morphological data because we do not understand the relationships among various morphological features and different biological aspects (*e.g.,* feeding, reproduction, locomotion). The disagreement among workers as to which nonmolecular features are phylogenetically important (see 19) is a testament to this uncertainty.

To avoid the problem of nonindependence, I have examined metazoan evolutionary relationships based on sequence data from the 18S nuclear ribosomal gene. With molecular sequence data, the determination of which characters are phylogenetically informative (as well as the specification of the character state) is much less subjective (see 20), and the characters can more easily be treated as independent. Even when characters (*i.e.,* nucleotide sites) are clearly not independent, the relationship and covariation between them are well characterized and can be compensated for (21).

Recent studies using 18S rDNA data have shown that either some (22, 23) or all (24) of the traditional lophophorate taxa are protostome organisms, and that the pterobranchs are hemichordates within the deuterostome clade (25, 26). Although these investigations suggest that the feeding apparatus is not congruent with known molecular homologies, I have combined the pterobranchs and the lophophorates into a single study (Table I) to allow a more rigorous examination of the issue. The entire 18S rDNA gene was aligned for all of the taxa with the exception of the *Rhabdopleura normani* sequence for which only the 5′ third of the 18S gene has been obtained. The remaining two-thirds was coded as missing (*i.e.,* as "N"), which allows these sites to be used for determining the relationships among the other taxa. Regions that could not be unambiguously aligned were excluded from subsequent analyses, and nucleotide sites were considered as independent characters (20). The

Table I

Taxa used in phylogenetic analysis

Species	GenBank acc. no.	Taxon
Plumatella repens	U12649	Bryozoan
Terebratalia transversa	U12650	Articulate
Glottidia pyramidata	U12647	Inarticulate
Phoronis vancouverensis	U12648	Phoronid
Rhabdopleura normani	U15664	Pterobranch
Balanoglossus carnosus	D14359	Enteropneust
Antedon serrata	D14357	Crinoid
Asterias amurensis	D14358	Seastar
Artemia salina	X01723	Crustacean
Placopecten magellanicus	X53899	Bivalve
Branchiostoma floridae	M97571	Cephalochordate
Xenopus laevis	X04025	Vertebrate
Acanthopleura japonica	X70210	Chiton
Tenebrio molitor	X07801	Insect
Anemonia sulcata	X53498	Cnidarian
Sycon calcaravis	D15066	Sponge

alignment can be obtained from the author at internet address *Halanych@mail.smu.edu.*

The alignment of the 18S rDNA sequences produced 1608 unambiguously aligned positions of which 295 were phylogenetically informative characters (*i.e.,* parsimony sites or shared derived characters). The general heuristic search algorithm of PAUP (*v.* 3.1.2d5; 27) and equal character weighting generated six equally parsimonious trees (1041 steps). The consistency index (C.I.) was 0.667 (C.I. = 0.561 when excluding uninformative characters), indicating that there was not a large amount of homoplasy among the data. Of the six trees, the one shown in Figure 1A conforms most closely to the traditional phylogeny (3, 10) because the deuterostomes are monophyletic. A maximum likelihood analysis (using PHYLIP *v.* 3.5; 28) revealed that none of these topologies are significantly worse than the best tree (likelihood score of −7753.43979). Figure 1B shows the strict consensus of these six trees.

The phylogenies recovered are consistent with the finding that bryozoans, brachiopods, and phoronids are protostomes, and that the pterobranchs are deuterostomes. The pterobranchs fall outside both the inclusive bryozoan–brachiopod–phoronid node and the protostome node; these have bootstrap values of 84% and 71%, respectively (Fig. 1B). Bootstrap values ≥70% usually correspond to a probability of ≥95% that the given clade is real (29). When the empirically derived transition–transversion ratio (1.4:1) is used to weight character changes accordingly, the bootstrap analysis yields values of 90% for the bryozoan–brachiopod–phoronid node and 76% for the protostome node. Transition–transversion ratios of 2:1, 3:1, and 10:1 give similar results. Additional analyses in which only the first third of the 18S gene (which is available for all taxa) was used also show that the bryozoans, brachiopods, phoronids, and molluscs form a monophyletic clade to the exclusion of the pterobranchs (bootstrap value of 88% for equal weighting and 94% for weighting transversions 1.4:1 over transitions). The phylogenetic analysis of the 18S rDNA data is therefore inconsistent with the putative homology of the feeding apparatuses.

Not only do the molecular data argue against homology, but recent fossil evidence also suggests that the lophophorates and pterobranchs are not closely related. Conway Morris and Peel (30) propose that extinct metazoans known as halkieriids are actually the common ancestor of annelids and brachiopods, and are closely related to molluscs. More evidence is needed to determine the exact position of the halkieriids, but the paleontological evidence (30) definitely places at least one of the lophophorate groups, the brachiopods, within the protostome lineage. The pterobranchs, on the other hand, are closely related to graptolites (31) and are clearly deuterostomes.

As for the morphological data based on extant taxa, the position of the lophophorates relative to pterobranchs is dependent upon which features are considered phylogenetically informative (32). Rigorous cladistic analyses (9, 33) suggest that lophophorates and pterobranchs form a basal paraphyletic grade within the Deuterostomia; *i.e.,* these taxa delineate a group which includes some, but not all, descendants of a common ancestor near the root of the deuterostome clade. Some of the characters employed in these analyses may, however, be ecological, and not phylogenetic, in nature; *e.g.,* the chaetognaths have been shown not to be in the Deuterostomia despite the fact that their blastopore is retained to form an anus (34, 35), and some potentially important characters—for example, the similar setae of annelids and brachiopods—are occasionally overlooked. Ideally, a phylogeny based on morphology should be developed by reanalyzing the existing data as more information becomes available. In reality, this is a somewhat subjective undertaking because it is difficult to agree on which morphological features are phylogenetically informative. Although morphological data are definitely an important phylogenetic tool, their application is not always useful or appropriate (36).

To date, no rigorous phylogenetic analysis has produced results suggesting that the pterobranchs, brachiopods, phoronids, and bryozoans form a monophyletic taxon rather than a paraphyletic grade. On the basis of the molecular and paleontological data, the criterion of congruence clearly is not satisfied. Thus, Patterson's three-criterion test for homology is failed. The most parsimonious explanation is that a lophophore-like apparatus has evolved at least twice in metazoans, and the homology, advocated by previous workers (3–8), between the lophophorate and the pterobranch feeding apparatuses is refuted.

Given this result, the degree of convergence in the feeding apparatus of these taxa is particularly striking. These organisms are ultrastructurally and mechanically very

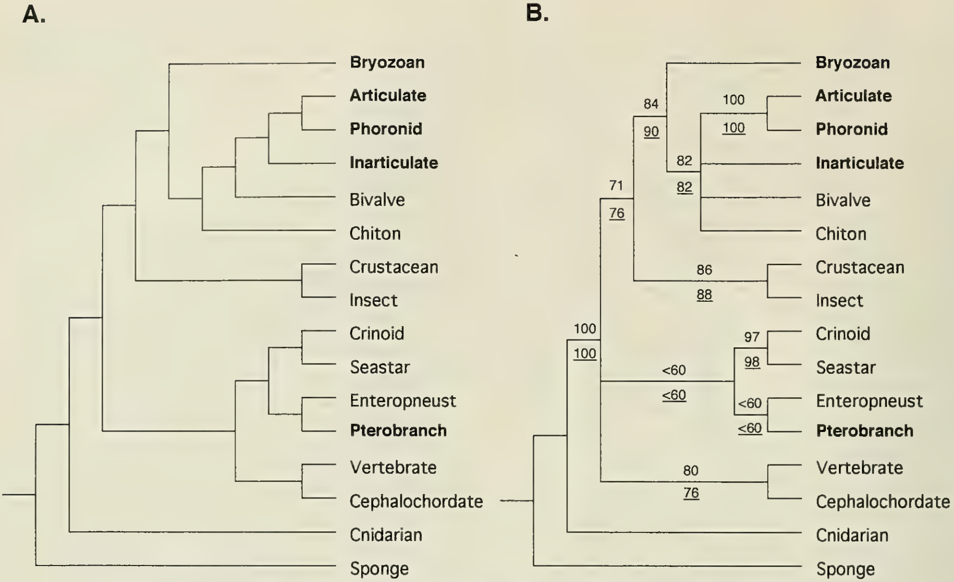

Figure 1. (A) The most traditional (in that the deuterostomes are monophyletic) of the six trees produced from the general heuristic search of PAUP. The trees had a length of 1041 with a consistency index of 0.667 (C.I. = 0.561 excluding uninformative characters). The g_1 statistic ($g_1 = -1.096090$) indicates that there is significant phylogenetic signal in this data set. (B) The strict consensus tree produced from the six topologies obtained in the heuristic search. The pterobranchs and lophophorates are several nodes apart. The values along the branches are bootstrap values (plain numbers are equal weighting and underlined numbers are transversions weighted 1.4 times transitions) produced from 500 iterations of a heuristic bootstrap analysis. All collapsed branches in the strict consensus topology were supported in less than 60% of the bootstrap iterations.

similar, which presumably reflects convergence due to comparable selective pressures. Bryozoans, brachiopods, phoronids, and pterobranchs are all sessile organisms that feed on unicellular algae about 7–20 μm in length. Conceivably, physical characteristics (*e.g.*, viscosity) that affect the capture and manipulation of particles in this size range, combined with a sessile existence, exert a strong selective influence toward a similar morphology. Obviously, however, a lophophore-like apparatus is not the only approach to suspension-feeding for a sessile animal. Bivalves, polychaetes, and urochordates use completely different mechanisms.

Despite the amazing similarity among the feeding apparatuses of the traditional lophophorates and pterobranchs, there are a few differences. For example, the feeding apparatus in pterobranchs does not surround the mouth as completely as it does in the lophophorates. Also, monociliated cells make up the tentacular ciliary bands in pterobranchs, brachiopods, and phoronids, but bryozoans have multiciliated cells. Thus a closer inspection of the feeding apparatus in different lineages may reveal that the similarity is more superficial than believed. Unfortunately, because the information is not complete for some groups (*e.g.*, rhabdopleurid pterobranchs), a more rigorous analysis at the electron microscopy level is necessary to determine the degree of similarity. Ideally, this analysis would consider all the taxa simultaneously.

The hypothesis that a lophophore-like apparatus has arisen at least twice in metazoan evolution raises some interesting evolutionary questions. How is the pterobranch feeding apparatus and ciliation similar to an enteropneust's proboscis and collar? Given that the lophophorates are protostomes, why is their ciliation pattern so different from that of other sessile suspension-feeding protostomes? Was a lophophore a primitive protostome character? Before many of these questions can be answered, we need a more complete understanding of protostome relationships. In view of the recent placement of the lophophorates suggested by molecular and fossil data, the relationships between annelids, molluscs, lophophorates, and other protostome taxa are not clear. Thus, to answer the above questions, to determine whether the traditional lophophorates are polyphyletic or paraphyletic, and to determine if brachiopods are polyphyletic (as indicated by the molecular data in Fig. 1), additional taxa must be sampled for both molecular and morphological data.

Finally, the convergence in feeding morphology in pterobranchs and lophophorates suggests that the evolutionary trajectory of major metazoan lineages, such as subkingdoms or phyla, can be very similar despite distinct phylogenetic origins.

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Karyotype of the Sea Anemone *Aiptasiomorpha* sp. (Anthozoa, Actiniaria) From Japan

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In the Anthozoa, only one karyological study has been performed, namely, a study of the coral Goniopora lobata *(2n = 28) by a "squash" method (1), and no information about the chromosomes of a sea anemone has been presented apart from a recent preliminary report of the diploid chromosome number and the complement of chromosomes of* Haliplanella lineata *(=* H. luciae*) (2n = 32), which were examined by an air-drying method (2). Morphological details of the chromosomes were not provided.*

The present study is the first attempt at karyotypic analysis in Actiniaria, a poorly studied order of Coelenterata (3–6). The karyotype of Aiptasiomorpha *sp. was determined by conventional Giemsa-stained preparations of early embryos. This species has 2n = 32 chromosomes, consisting of 14 pairs of biarmed chromosomes and 2 pairs of chromosomes having one arm or with the second arm extremely small. The presence of two large metacentric pairs characterizes the karyotype of* Aiptasiomorpha *species.*

Specimens of *Aiptasiomorpha* sp. were collected from the coast at Hashirimizu, Kanagawa Prefecture, near Tokyo. The specimens were identified by Dr. H. Uchida (Sabiura Marine Park Research Station). In an aquarium, this small acontiate sea anemone reproduces asexually, by pedal laceration, with great frequency. Moreover, it was found as a dioecious form in the present location. Larvae developed externally after the synchronous release of eggs and sperm. Spawned eggs, suspended in a small amount of seawater, were inseminated, and the fertilized eggs were cultured in the laboratory (room temperature at 25°C). The early development of *Aiptasiomorpha* sp. (author, unpub. data) resembles that reported by Fukui for *H. lineata* (7). In this study, chromosome preparations were made from embryos at the early cleavage stages; there was no need to remove the fertilization membrane, yolk granules, or oil droplets. To obtain well-spread chromosomes, the embryos had to be adequately dissociated.

Conventionally prepared Giemsa-stained chromosomes of *Aiptasiomorpha* sp. are shown in Figure 1. The chromosome number was determined as 2n = 32 on the basis of 84 well-spread metaphase plates. The presence of two

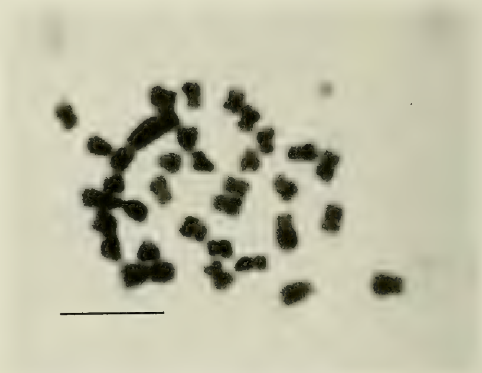

Figure 1. A typical metaphase spread of *Aiptasiomorpha* sp., consisting of 32 chromosomes. Chromosome preparations were made from embryos at the 2- to 16-cell stage. Embryos were kept alive for 30 min in a 0.025% solution of colchicine, then treated with hypotonic solution (0.05 *M* KCl) (12) for 10 min at room temperature and fixed immediately in a mixture of acetic acid and absolute ethanol (1:3, v/v). To make slides for chromosome preparation, a method used by Rahat *et al.* (6) in their study of hydra chromosomes was applied with some modifications. A cell suspension in fixative was transferred into 60% acetic acid and dried. Spread cells then stained for 30 min with a 20% Giemsa solution. Bar represents 10 μm.

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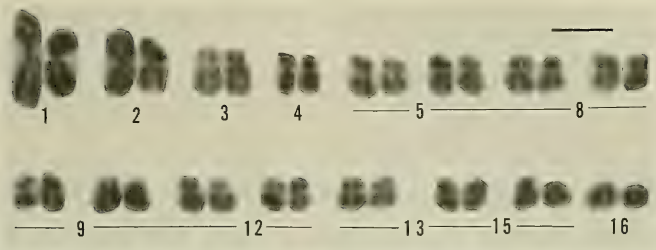

Figure 2. A conventional Giemsa-stained karyotype of *Aiptasiomorpha* species. Bar represents 5 μm.

large pairs, which account for nearly 25% of the total length of all the chromosomes, is characteristic of chromosomes in this species. Figure 2 shows that the karyotype consists of 16 pairs of homologous chromosomes that can be divided into three groups according to their size. Chromosome pairs 1 and 2 are distinctly larger than the remainder. The two pairs belonging to the group of large chromosomes are metacentric elements. Of the medium pairs 3–12, only one pair has a very small arm, and the others are biarmed; pair 3 is regarded as submetacentric and pair 4 as metacentric. The small pairs 13–16 consist of three biarmed and one-armed elements; pair 16 is regarded as telocentric. Because their sizes vary gradually, the distinction between the group of medium chromosomes and small chromosomes is not clear.

The genus *Aiptasiomorpha* belongs to the subtribe Acontiaria. Although the taxonomic treatment differs slightly among authors, other genera, such as *Diadumene* or *Haliplanella,* are often included with *Aiptasiomorpha* as members of the family Diadumenidae (8, 9). But the relationships of the three genera in the family are not definitely known at present (10, 11). Thus, *Aiptasiomorpha* sp. and *H. lineata* are considered to be taxonomically close to one another. However, the members of the two species differ in size: *Aiptasiomorpha* sp. is very small and has been confused with immature forms of *H. lineata.* At Hashirimizu, *H. lineata* is one of the most abundant species found in the intertidal region along the coast, whereas populations of *Aiptasiomorpha* sp. can be extremely well circumscribed within the same area. It will be of interest to clarify the phylogenetical relationship between these two sea anemones.

With respect to the general features of the chromosomes, as studied by conventional Giemsa staining, the two Japanese acontiate sea anemones—*Aiptasiomorpha* sp. in the present study and *H. lineata* in previously published studies—have the same chromosome number but very different chromosome morphology. Namely, the two larger chromosome pairs in *Aiptasiomorpha* sp. are metacentric, but the six larger pairs in *H. lineata* seem to be one metacentric and five telocentric pairs. Further detailed karyological analysis, including banding studies, will be the subject of future research. Banding analysis has apparently not been applied to date to any numbers of Coelenterata. With slight modification, the methods employed in this report might make it possible to apply standard banding techniques in future analyses.

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Embryos at the Edge of Tolerance: Effects of Environment and Structure of Egg Masses on Supply of Oxygen to Embryos

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Abstract. Oxygen concentrations in gelatinous egg masses of two species of opisthobranch gastropods were examined with microelectrodes. Embryos in central positions are near the limit of the oxygen supply required for development. This limit is approached despite a diffusion constant for oxygen in masses that is close to that in water. Closed-chamber respirometry shows that oxygen is consumed by masses in the dark but generated in the light. Internal oxygen concentrations were greater in bright than in dim light. Thus photosynthesis and respiration of microorganisms associated with the masses affects the supply of oxygen to embryos within the mass. This effect of light was confirmed for egg masses of a polychaete. These observations, together with other published observations on the effects of hypoxia on development, indicate that the developmental rates of embryos in egg masses may depend on algal photosynthesis and metabolism. Flow around the masses also affects delivery of oxygen to embryos, but masses in dim light are at the limit of adequate supply even in a strong flow with a very thin boundary layer. Because the central embryos are near the limit for adequate supply of oxygen by diffusion, their development rate thus depends on light, abundance of photosynthetic and heterotrophic microorganisms, flow, and oxygen concentration in the surrounding water.

Introduction

Supply of oxygen limits the rates of development of crowded embryos with limited diffusional exchange (Strathmann and Strathmann, 1995). Retarded development of internal embryos suggests that egg masses of diverse taxa approach the limits of adequate diffusional exchange (Chaffee and Strathmann, 1984; Giorgi and Congleton, 1984; Lucas and Crisp, 1987; Seymour and Roberts, 1991). Such limits could affect the size and form of clutches and the adequacy of sites for deposition of egg masses. Models of supply of oxygen by diffusion suggest that many egg masses are near the limits of thickness of mass and concentration of embryos (Crisp, 1959; Strathmann and Chaffee, 1984; C. E. Lee and R. R. Strathmann, in prep.). Adequate gas exchange for embryos might even influence evolution of alternative modes of development, with dispersal of embryos in the plankton being a simple means of ventilating large clutches of embryos (Strathmann and Strathmann, 1995).

Other environmental factors also impose functional constraints on an egg mass. Functional requirements for the retention and protection of embryos may explain many features of masses. The constraints associated with supply of oxygen and demand for oxygen nevertheless set limits on the structure of clutches. Understanding the physiological limits on egg masses is one step toward understanding the diversity within these limits.

The study described here had two objectives. The first was to test assumptions of models for limits on aggregations of embryos that are supplied with oxygen by diffusion. These assumptions include (1) diffusion constants close to that for oxygen in water, (2) oxygen gradients that approach an oxygen concentration of zero at the center of masses of embryos, and (3) rates of oxygen consumption close to those for hatched larvae. The models predict limits on the structure of clutches: the size and form of

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the mass and the concentration of embryos. Are the assumptions approximately correct?

The second objective was to examine the effects of fouling on the supply of oxygen to embryos. Many egg masses are fouled by unicellular algae and other microorganisms. Algal photosynthesis in light and respiration in darkness can have a profound effect on the supply of oxygen to the embryos of some amphibians (Bachmann *et al.,* 1986). Scaling models for the thickness of clutches and concentrations of embryos in clutches have considered only the respiration of the embryos (Strathmann and Chaffee, 1984; Seymour and Bradford, 1995). If the thin films of fouling microorganisms that are common on egg masses have large effects on oxygen supply, then scaling of egg masses is sensitive to fouling and light intensities as well as to such internal constraints as the concentration of embryos, oxygen consumption by embryos, and the diffusion constant for oxygen.

Materials and Methods

Egg masses

Egg masses of the cephalaspidean opisthobranch gastropods *Melanochlamys diomedea* (Bergh) and *Haminaea vesicula* Gould were used for most observations. Some observations were confirmed with egg masses of the nereid polychaete *Nereis vexillosa* Grube. The egg masses were either collected from False Bay and Argyle Lagoon, San Juan Island, Washington state, or deposited in the laboratory by adult cephalaspideans collected from those locations. Before use, masses were maintained in continuous flow aquaria or in aerated beakers at 12° to 15°C, which was 1°–2°C above the temperature in tidally mixed channels.

The egg masses of *M. diomedea* and *H. vesicula* are described by Hurst (1967) under the names of *Aglaja diomedea* and *Haminoea virescens* and by Strathmann (1987). The egg masses of *M. diomedea* are elongated spheroids often about 1 cm or less through the short axes and greater than 1 cm in the long axis, with a tether at one end that extends into the sediment. The egg masses of *H. vesicula* are thin ribbons about 2 mm thick and 8 mm broad; they are attached at one edge to aquatic plants, shells from dead bivalves, driftwood, or other surfaces. The egg masses of both species are often found intertidally in pools on sand flats, where temperatures at low tide can reach 25°C, or in shallow lagoons. These masses are at times within a few centimeters of the water's surface, exposed to full sunlight.

As in other opisthobranchs, the embryos are in small fluid-filled capsules embedded in a gelatinous matrix. In *H. vesicula* and *M. diomedea* each capsule contains one embryo or occasionally two, with sufficient room for the embryos to rotate once they develop cilia.

The egg masses of both species were sufficiently regular to be measured to the nearest 0.5 mm with calipers. The thinner egg mass of *H. vesicula* has a higher concentration of embryos, about 70,000 g^{-1} in contrast to about 23,000 g^{-1} for *M. diomedea* (C. E. Lee and R. R. Strathmann, in prep.). Eggs of the two species are similar in size, with reported diameters of 75 and 90 μm for *H. vesicula* and 83 and 98 μm for *M. diomedea* (C. E. Lee, pers. com.; Strathmann, 1987).

The egg masses of *Nereis vexillosa* are described by Johnson (1943). They are irregular, several centimeters across, but divided by lobes and channels. Because of the irregular positions and sizes of lobes and channels, minimum diffusion distances are not easily measured, but embryos within a lobe are as much as several millimeters from the surface of the lobe (Chaffee and Strathmann, 1984), and a whole mass is several centimeters in diameter. The masses lie on the substratum unattached and are often stranded intertidally.

Apparatus

Different types of electrodes were available at different times during the study. A few observations were made with a microelectrode with external reference (Diamond General), but most were with micro-Clark electrodes (Diamond General Corp., Ann Arbor, Michigan, and Micro-Sense, Ramat Gan, Israel) with tip diameters of 100 or 150 μm. All electrodes were polarized by a Chemical Microsensor meter (Diamond General Corp.) connected to a chart recorder for permanent recording of data. All electrodes were calibrated in seawater at zero percent oxygen and at air saturation for a given temperature. Most zero calibrations were in nitrogen-bubbled seawater; some were in anoxic sediment with pablum (baby cereal) added to maintain anoxia. A few zero calibrations of the electrodes with external reference were with a 2% solution of sodium sulfite, but this method is incompatible with electrodes that have an internal reference. Zero and saturation calibrations were done at the beginning and end of each recording session to check for drift in electrode sensitivity. Drift was seldom a problem, and data were not used unless drift was negligible.

Stirring sensitivity was determined by the difference between readings in gel and in stirred water at air saturation. The differences between air saturation and zero oxygen were adjusted accordingly for measurements within gelatinous masses. With the Diamond General external-reference electrodes, the apparent mean drop in oxygen was 8.3% across the surface of gelatinous masses of *M. diomedea* at air saturation. With the Diamond General micro-Clark electrodes, the apparent mean drop in oxygen because of stirring was 4.2% at air saturation. No effect of stirring was detected for the Micro-Sense electrodes.

The chambers for measurements with microelectrodes were surrounded by a water bath to keep temperature constant. The chamber for most observations was a beaker submerged in a temperature-controlled water bath. Gas bubbled to maintain concentrations in solution was passed through a coil within the bath to maintain a constant temperature. Egg masses were held in a stream of gas bubbles to minimize the thickness of the boundary layer. The other chamber was a recirculating flow tank with a water jacket to maintain temperature. Most measurements were at 20°C, which is well within the range encountered in nature. A few measurements were made at the temperature of seawater in the aquarium system, which is lower and approximates temperatures at field sites at high tide.

Electrode tips were marked with evenly spaced black lines so that the position of the tip of the electrode could be determined for measurement of oxygen profiles through egg masses.

Measuring gradients

Gradients of oxygen concentration were measured in the globose masses of *Melanochlamys diomedea* with an electrode inserted into the mass along a radius from the outer surface toward the center along the short axis of the mass.

Oxygen concentration at the center of a mass was determined by recording continuously as the electrode was moved into the mass. Concentration was lowest at about the center of a mass, and this value was taken as the central oxygen concentration. Each paired comparison of central and peripheral oxygen concentration was made with a different mass.

Measuring the diffusion constant

The diffusion constant was estimated for the spheroidal masses of *M. diomedea.* The embryos and associated microorganisms were killed by microwaving the masses in a much larger volume of seawater for 8 min to temperatures approaching 60°C. The optical properties of the gel did not change discernibly from this heating. Subsequent microbial growth was controlled by 40 to 100 μg ml^{-1} of gentamycin in seawater (Leahy, 1986). Each mass was equilibrated with nitrogen-purged water and then reequilibrated to air-saturated water for diffusion of oxygen into the anoxic mass, and each mass was also equilibrated with air-saturated water and then reequilibrated to water purged of oxygen by nitrogen bubbling for diffusion of oxygen out of the mass. The diffusion constant was estimated by using the equation of Ingersoll *et al.* (1948, pp. 162–164 and appendix H). This was the method used by O'Dor and Balch (1985) to calculate heat transfer in a spherical egg mass. The equation is

$$(C_c - C_s)/(C_o - C_s) = 2[\exp(-\pi^2Dt/R^2) - \exp(-4\pi^2Dt/R^2) + \exp(-9\pi^2Dt/R^2) - \cdots]$$

with C_c, C_s, and C_o the central, surface, and initial concentrations of oxygen, D the diffusion constant, t time, and R the radius of the sphere. We took the two short axes of the mass as the diameter of a sphere, an assumption that should underestimate the diffusion constant. Estimates of diffusion constants for a mass were consistent except near the asymptote of equilibration, where small errors in measurement of oxygen magnify errors in calculated diffusion constants. We therefore calculated diffusion constants with a reading of central oxygen concentration taken when the oxygen concentration was changing rapidly and was far from the initial and final equilibrium values. The times used were 0.75 or 1 h from the start of each run toward a new equilibration.

Effect of light

To examine the effect of light on oxygen concentrations within masses, we used a halogen lamp with fiber optics and a heat filter (2.5 cm of a solution of copper sulfate) to eliminate temperature changes. Although differences in direction and spectrum of light prevent exact comparisons between light in the laboratory and field, we tried to achieve an intensity of light similar to full summer sun at the water's surface. Readings from a photographic light meter for the arrangement with the fiber-optic light were close to readings from a cloudless summer sky. Black plastic sheets provided darkness. Dim light was the illumination from overhead fluorescent lights in the laboratory, the condition for most of our measurements of oxygen in egg masses. For measurements of effects of light on oxygen concentrations in the cephalaspidean egg masses, the light conditions were maintained until the oxygen concentration became constant, and then oxygen concentration was noted. The cephalaspidean masses were held in the temperature-controlled chamber in a stream of air bubbles. The masses of *Nereis vexillosa* were held in air-saturated water in a flow tank.

Consumption and production of oxygen were determined with a Clark-type oxygen electrode and a closed, well-stirred chamber of 4.1 ml, as described in Herbert and Waaland (1988). Consumption by the electrode was too small to be measured within intervals of several hours. The slope of a chart recording of oxygen concentration over time gave the rate of production or consumption. The concentration of oxygen was calculated from the solubility of oxygen in seawater at 1 atm, about 5.3 μl ml^{-1} at 20°C (Carpenter, 1966). A lamp in a slide projector provided visible light on egg masses at an intensity about half that of summer sun (about 1000 μmol photons m^{-2} s^{-1}) (Herbert and Waaland, 1988).

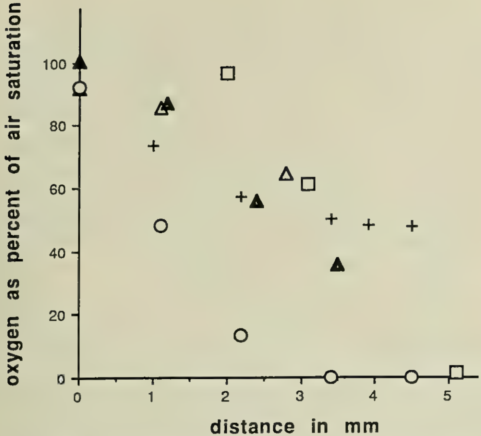

Figure 1. Oxygen concentrations from the surface toward the center along the diameter of five egg masses of *Melanochlamys diomedea* at 20°C. The stages of the masses were early cleavages (+), blastulae (△, open and filled), gastrula–trochophore (□), and veligers (○). The diameters, in the same order, were 9, 10, 8, 11, and 9 mm.

Following estimates of oxygen consumption for masses of *M. diomedea,* numbers of embryos were counted by freeing capsules from the gel with a 1% solution of sodium hypochlorite. The capsules were rinsed, and a suspension of capsules from a mass was subsampled for counts of embryos. The accuracy of subsampling was confirmed by a few counts of capsules from whole masses.

Results and Discussion

Gradient of oxygen concentration in masses

Oxygen concentrations in egg masses declined from the surface toward the center. The gradient in oxygen concentration was most easily and clearly demonstrated for masses of *Melanochlamys diomedea,* which were regular in shape and sufficiently thick that an electrode could be placed at measured distances from the surface toward the center (Fig. 1). The oxygen concentrations were similar near the surface in masses at all stages of development. Oxygen concentrations declined more rapidly from the surface toward the centers of masses at more advanced stages of development. Variations in oxygen concentration that were unrelated to stage of development could have resulted from variation in size of masses, concentrations of embryos, fouling by microorganisms, or errors in estimating the position of the electrode tip. For example, the near-saturation value at 2 mm in one mass at the gastrula–trochophore stage (Fig. 1) could have been due to an unusually thick embryo-free zone near the outer surface or an error in estimating the depth of the electrode tip.

To test for the significance of the effects of stage of development and distance from the surface, we used ANOVA for the outer and nearly linear portion of the oxygen gradient, eliminating the inner three points for the mass at early cleavage stages and the inner two points for the mass at the veliger stage. Distance from the surface, stage of development, and their interaction were all significant at $P < 0.01$ ($n = 15$, four stages, two masses at blastula stage lumped). This result was also obtained when the two masses at the blastula stage were treated as separate stages. The significant interaction confirmed the steeper gradient in the outer part of the egg mass at more advanced stages of development (Fig. 1).

Oxygen concentrations at centers of masses

Oxygen concentrations were extremely low at the centers of masses. Central oxygen concentrations were lower in masses at more advanced stages of development (Fig. 2). Central oxygen concentrations were low in both the thin and thick masses but lower in the thicker globose masses of *Melanochlamys diomedea* than in the thinner ribbons of *Haminaea vesicula* (Fig. 2). In an ANOVA with stage as a continuous variable, the effects of stage and species on central oxygen concentration were signif-

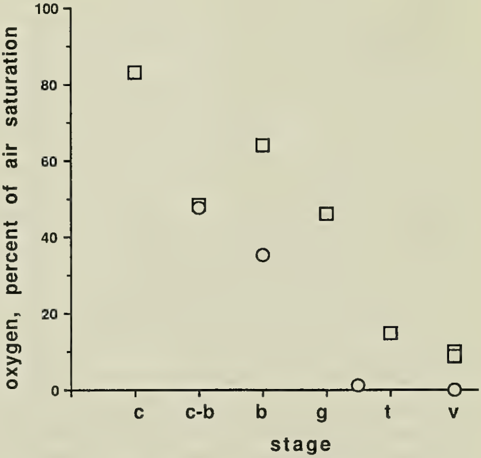

Figure 2. Oxygen concentrations at the centers of gelatinous egg masses versus stages of development. Stages are early cleavages (c), cleavages to blastula (c–b), blastula (b), gastrula (g), trochophore (t), and veliger (v). Circles are masses of *Melanochlamys diomedea;* squares are masses of *Haminaea vesicula.* Moving cilia develop between the gastrula and trochophore stages.

Table I

Estimated diffusion constants for oxygen in three egg masses of Melanochlamys diomedea *based on the equation for diffusion in a sphere of diameter equal to the two short axes of the masses*

Axes (mm)	Diffusion constant* (10^{-5} cm^2 s^{-1})	Estimated depths of electrode tip (mm)	Directions of equilibration
13 × 11.5 × 11.5	2.14, 2.01, 1.91	5.6, 5.1, 4.5	++−
19 × 9 × 9	1.92, 1.87	4.5, 4.5	+−
17.5 × 8 × 8	1.37, 1.51	4.5, 4	+−

* Each estimate is for a separate equilibration with directions of equilibration anoxic to air saturated (+) or air saturated to anoxic (−).

icant at $P < 0.001$ and $P = 0.021$ ($n = 11$, df 1, 8). The interaction term was omitted from the test because it was not significant in an ANOVA that included it. The diffusion distances were much less for the egg ribbons of *H. vesicula* (about 8 to 10 mm wide and 2 mm thick) than for the globose masses of *M. diomedea* (13 to 16 mm long and 8 to 11 mm in diameter). However, the concentration of embryos was much higher in ribbons of *H. vesicula* (see above). Central oxygen concentrations were low even though these masses were held in a stream of air bubbles so that water near the surface of the mass was air-saturated and well-stirred. All of these masses were at 20°C.

Diffusion constant

Diffusion constants for oxygen in egg masses were estimated from the rate of change in oxygen concentrations at the centers of egg masses first equilibrated at one oxygen concentration and then exposed to water at a different oxygen concentration. The reequilibration was from air saturation to anoxia or from anoxia to air saturation. Three egg masses from *M. diomedea,* in which embryos and microorganisms had been killed, were reequilibrated in both directions for at least two observations per mass, in all cases at 20°C. The estimates of the diffusion constant (Table I) were close to the 2×10^{-5} cm^2 s^{-1} reported for water at 20°C (Horne, 1969). There was little variation between estimates from separate equilibrations for an egg mass, but significant differences between estimates for different egg masses (ANOVA, $P < 0.01$).

This result indicates that the low supply of oxygen to central embryos is not the result of a low diffusion constant; however, several assumptions of the calculation could bias the estimate. We assumed that the masses were spheres with diameters equal to the short axes of the masses. The longer diffusion distance in the long axis should result in an underestimate in the diffusion constant, and the estimated diffusion constants were indeed lower for the two elongate masses than for the more nearly spherical mass (Table I). Misplacement of the electrode off-center should have the opposite effect, but repeat runs gave similar values even when the electrode was replaced at depths differing by up to 1 mm. The estimate of diffusion constant is sensitive to measurements of the diameter of the mass, and the inaccuracy of measuring the dimensions of gelatinous masses with calipers could bias estimates of the diffusion constant. Distortion and wobbling of the mass in the bubble stream could shorten diffusion distances, thus giving an overestimate of the diffusion constant. The estimates of the diffusion constant are approximate but support an assumed value close to the diffusion constant for oxygen in water.

Effect of light on oxygen concentration

Gelatinous masses become fouled with a variety of organisms. Diatoms were especially conspicuous among foulers of the opisthobranch and polychaete masses. Respiration and photosynthesis by these fouling organisms would be expected to affect net oxygen consumption by the whole mass, thereby affecting the supply of oxygen to internal embryos. We tested this hypothesis by measurements of oxygen concentrations in bright light in contrast to dim light or darkness. Oxygen concentrations within egg masses were affected by light (Table II). In the masses of the opisthobranchs, for which fouling was restricted to the surface, oxygen concentrations were affected both at the surface of the masses and at depth. Under some conditions the fouling organisms produced supersaturating amounts of oxygen near the outer surface, even in well-stirred and air-saturated water, as in the mass of *H. vesicula* with oxygen at 130% of air saturation. The egg masses of *Nereis vexillosa* were conspicuously fouled by diatoms and other organisms, which penetrated into the mass. Because the shapes of these egg masses were irregular with lobes and channels, the position of the electrode relative to the surface of the mass was not so easily char-

Table II

Effect of light on oxygen inside egg masses of Melanochlamys diomedea, Haminaea vesicula, *and* Nereis vexillosa

Species	Stage	Position of electrode	Percent of air saturation		Ratio of concentrations in light and dark
			Dark or dim light	Bright light	
M. diomedea	veliger	2.2 mm inside	15.2	20.5	1.35
M. diomedea	veliger	near surface	38.1	47.5	1.25
H. vesicula	veliger	center	8.9	41.0	4.6
H. vesicula	veliger	near surface	—	130	—
N. vexillosa	with cilia	in a lobe	26	42	1.6
N. vexillosa	with cilia	in a lobe	21	49	2.3

Table III

Production and consumption of oxygen in a closed chamber by four masses of Melanochlamys diomedea *in light and dark*

Production in light (μmol O_2 min^{-1})	Consumption in dark (μmol O_2 min^{-1})	Volume of mass (mm^3)	Stage	Temperature (°C)
0.0048	0.0035	630	veliger	20
0.0065	0.0068	600	cleaving	20
0.0049	0.0030	271	hatching	20
0.0024	0.0040	352	veliger	12

Table IV

Consumption of oxygen per egg mass and per embryo for four masses of Melanochlamys diomedea *in a closed chamber in the dark; all were at early veliger stage*

Consumption (μmol O_2 min^{-1})	Volume of mass (mm^3)	Embryos per mass	Consumption per embryo (10^{-3} μl h^{-1})	Temperature (°C)
0.0037	527	14960	0.33	20
0.0052	471	18390	0.38	20
0.0059	670	9139	0.87	20
0.0047	452	23910	0.26	20
0.0029	452	23910	0.17	12

acterized, but light had a pronounced effect on oxygen concentrations within the mass.

Oxygen production and consumption in light and dark

Fouled masses of *M. diomedea* produced oxygen in bright light (Table III, Fig. 3). Microbial photosynthesis can be large relative to respiration by embryos. In the dark, the masses consumed oxygen (Table III, Fig. 3).

In the dark, the mean estimated respiration per embryo at 20°C was 0.46×10^{-3} μl h^{-1} (Table IV), which is close to that expected for hatched and swimming veligers of similar size (Bayne, 1983). This agreement may not reflect real similarity in the respiration rates of hatched and unhatched larvae. Instead, respiration by microorganisms may be inflating the oxygen consumption per unhatched veliger.

Oxygen consumption per embryo was calculated from consumption by whole masses, and the variation in estimates could therefore be a result of differences in fouling by microorganisms. The highest estimate (0.87×10^{-3} μl oxygen h^{-1}) exceeds that expected for hatched veligers of similar size (Bayne, 1983), and respiration by microorganisms may account for the excess. However, the oxygen consumption per embryo is inversely related to the estimated concentrations of embryos in the masses listed in Table IV. Possible explanations for this relationship are that (1) it occurred by chance, (2) higher concentrations of embryos depressed oxygen consumption, or (3) egg masses with fewer embryos per unit volume had more fouling organisms per embryo. We have no reason to suspect large errors in counts of embryos.

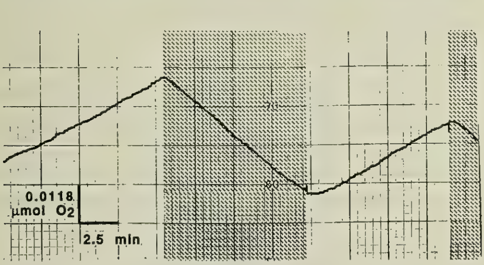

Figure 3. Trace of oxygen concentration in a closed chamber containing an egg mass of *Melanochlamys diomedea* in light and dark (interval shown by hatching).

Effect of boundary layer

An oxygen gradient around egg masses could be detected for egg masses in slow currents. For most of our observations, the boundary layer was minimized by holding masses in a stream of gas bubbles. To observe an external oxygen gradient around a mass, masses of *M. diomedea* were tethered in the sediments of a flow tank with a free-stream velocity of 8 cm s^{-1} at 18° to 19°C. Electrodes were positioned above the mass. Oxygen concentration dropped measurably as the electrode approached a mass (Table V), but the drop was much less than the decrease from the surface to the center of the mass (Figs. 1 and 2).

General Discussion

The measurements of oxygen concentrations within masses confirmed previous observations about the limits on the size and form of clutches of embryos. Oxygen concentrations were low near the center of embryo masses at

Table V

Gradients of oxygen concentration external to two egg masses of Melanochlamys diomedea

Axes of mass (mm)	Distance from mass (mm)	Oxygen (% of air saturation)
13 × 7 × 7	>10	85.0
	0 to 1	78.4
16.5 × 15 × 15	>10	100
	2	98.4
	1	96.5

the motile stages beyond gastrulation and in dim light. Even in air-saturated water, central embryos were near the oxygen concentration at which development is retarded (Strathmann and Strathmann, 1995). The measurements confirmed, approximately, assumptions on diffusion constants, oxygen gradients, and oxygen consumption that have been used to model the size and form of clutches (Strathmann and Chaffee, 1984). However, the observations also demonstrated that fouling microorganisms influence oxygen supply to embryos, a factor omitted from scaling models. We shall first discuss the properties of egg masses that influence oxygen supply and then discuss the effects of fouling microorganisms and other environmental factors.

The estimated diffusion constants for masses of *Melanochlamys diomedea* approach the diffusion constant for oxygen in water. Thus the low oxygen concentrations within the egg masses do not result from low diffusion constants for the material that separates and protects the embryos. The estimates for the diffusion constant are reassuring, because a diffusion constant close to that for oxygen in water has been a convenient assumption for models of oxygen supply to egg masses (Strathmann and Chaffee, 1984; Seymour and Bradford, 1995). The estimated diffusion constants were also close to Burggren's (1985) estimate of the diffusion constant for oxygen in frog egg jelly, which was 75% of that for oxygen in water. Our higher estimates for the diffusion constant surprised us because the diffusion constant in artificial gels decreases below that for water as the concentration of the polymer increases (Sato and Toda, 1983; Renneberg *et al.*, 1988). A possibility suggested to us by T. Hunter and P. Verdugo is that aligned macromolecules could allow more rapid diffusion in a gel. Another possibility is that biases in the measurement caused an overestimate, but the most obvious bias—the assumption that the masses were spheres of diameter equal to the two short axes—should underestimate the diffusion constant. A comparison of natural and artificial gel masses could determine whether the diffusion constants that we estimated for natural masses are indeed higher than for artificial gels of similar organic content or are in error.

Although features other than high diffusion constants may aid transport of oxygen to embryos, it is rate of diffusion that limits supply of oxygen for many clutches of embryos. Stirring by embryos in fluid-filled capsules may increase the rate of supply of oxygen (Burggren, 1985; Hunter and Vogel, 1986). However, embryos do not have motile cilia at early stages; stirring does not compensate for the increased metabolism at ciliated stages (Strathmann and Strathmann, 1995); and intracapsular stirring may be ineffective for transport through a mass if the oxygen concentration in each capsule is a local minimum (Seymour and Roberts, 1991). Pores that permit interstitial flow between embryos would impose a different kind of restriction on ventilation of masses (Strathmann and Chaffee, 1984). Although the gelatinous masses of amphibians have pores and the masses of *Nereis vexillosa* have irregular channels, most gelatinous masses lack pores.

The concentrations of oxygen at the centers of masses of *M. diomedea* were as low as those that retard development and cause smaller size at hatching (Strathmann and Strathmann, 1995). The observed concentrations were thus consistent with observations of retarded central embryos in masses from the field (Chaffee and Strathmann, 1984).

Even the thin ribbons of *Haminaea vesicula* were near the limit for a sufficient supply of oxygen to central embryos. The oxygen concentration was near 10% of air saturation at the center of the egg ribbon of *H. vesicula* when embryos were at the veliger stage in dim light. This oxygen concentration approaches the hypoxia at which development is retarded (Strathmann and Strathmann, 1995) despite the short diffusion distance to central embryos. Oxygen is depleted because the concentration of embryos in the ribbon is so high—much greater than in the thick globose egg mass of *M. diomedea*—about 70,000 g^{-1} compared with 23,000 g^{-1} (C. E. Lee and R. R. Strathmann, in prep.).

The discovery that the microorganisms fouling the masses played a large role in production and consumption of oxygen was in striking contradiction to the assumption made in previous models of diffusion in egg masses. Light increased oxygen supply to embryos by its effect on photosynthesis, and respiration by microorganisms must similarly decrease oxygen supply in darkness. Thus the limits on clutch structure that are set by oxygen supply cannot be predicted from the consumption of oxygen by embryos. Fouling microorganisms complicate predictions of scaling limits on the size, form, and concentrations of embryos in egg masses.

Light could switch masses from consumption of oxygen to production of oxygen. Deposition at sunnier sites could enhance oxygen supply by day, but respiration of microorganisms could decrease the supply of oxygen at night even at shallow sites. These effects of algal fouling have been observed for eggs of salamanders, with photosynthesis exceeding consumption in light and respiration severely depleting oxygen in darkness (Bachmann *et al.*, 1986; Pinder and Friet, 1994).

Visible algal fouling was limited to the surface of the masses of the opisthobranchs. For these masses, fouling changed the oxygen concentration at the boundary of the mass. In contrast, pennate diatoms and other fouling organisms penetrated the masses of *Nereis vexillosa* and changed the consumption and production of oxygen throughout the mass.

At high light intensities, damage from solar radiation could exceed the benefits from photosynthesis, but fouling algae could also protect embryos from solar radiation in the ultraviolet range. Solar radiation can be lethal for embryos in masses of some species (Biermann *et al.*, 1992; Blaustein *et al.*, 1994).

The concentrations of embryos and sizes of the masses examined here are near the limits at which the supply of oxygen supports full development rates and hatching sizes of central embryos (Strathmann and Strathmann, 1995). Other masses also approach these limits (C. E. Lee and R. R. Strathmann, in prep.). Photosynthesizing microorganisms, high oxygen concentrations in the surrounding water, and narrow boundary layers are factors that maintain an adequate supply of oxygen for development. Respiring microorganisms, low oxygen concentrations in the surrounding water, and weak velocity gradients around the masses tip the balance toward hypoxia and thereby retard or arrest development. These conditions influencing oxygen supply could affect the evolution of the size and shape of egg masses, the concentration of embryos, and the choice of deposition sites.

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Intersex Red Claw Crayfish, *Cherax quadricarinatus* (von Martens): Functional Males with Pre-vitellogenic Ovaries

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Abstract. Intersex individuals, possessing both male and female genital openings, were assessed in two groups–7 and 19 months old–of Australian red claw crayfish (*Cherax quadricarinatus*). All intersex individuals investigated were functional males, as suggested by their male-like morphology and the presence of testes, sperm ducts, androgenic glands, and viable spermatozoa. When an ovary was present in an intersex individual from either group, the gonadosomatic index, the diameter of the oocytes, and the ovarian cytosolic polypeptide profile were similar to those of immature, pre-vitellogenic females. We conclude that intersexuality in *C. quadricarinatus* does not indicate a case of protandric sequential hermaphroditism, as previously suggested. The case of intersexuality described here presents a unique model for the study of the role of the androgenic gland in the regulation of sex differentiation in crustaceans.

Introduction

Intersexuality among crustaceans has been documented mainly in species that exhibit either protandry (change of sex from male to female) or protogyny (change of sex from female to male). Protandry is predominant among hermaphrodite crustaceans, and within the Malacostraca it has been reported in nine families of Decapoda (Brook *et al.*, 1994). Two types of cases in which an individual possesses both male and female openings were described among decapod crustaceans. In one type (*e.g., Nephrops norvegicus*), the individual exhibits a complete bilateral separation: half of the body has male internal and external characters, and the contralateral half has only female characters (Farmer, 1972; Johnson and Otto, 1981; Chace and Moore, 1983). In other cases, including several species of Australian parastacides, the intersex individual possesses both male and female openings, but all the other external characters are male-like (Lake and Sokol, 1986; Sokol, 1988; Brummett and Alon, 1994). Intersex individuals may represent cases of true hermaphroditism, in which the androgenic gland disappears to permit the expression of the feminine phase in protandric species (Charniaux-Cotton, 1958). However, some cases of non-functional hermaphroditism in males of gonochoristic species were also described in malacostracans (Charniaux-Cotton and Payen, 1985).

The Australian red claw crayfish, *Cherax quadricarinatus* (von Martens), is a large, tropical freshwater crustacean that grows and reproduces successfully in temperate climates, attaining sexual maturity within 7 to 9 months (Rouse *et al.*, 1991). It is a gonochoristic species with a bilaterally symmetrical reproductive system. In males, this consists of a pair of testes, sperm ducts, androgenic glands, and genital openings at the base of the fifth walking legs. Females have a pair of ovaries, oviducts, and genital openings at the base of the third walking legs. Occasionally, intersex individuals with both male and female genital openings have been recorded (Thorne and Fielder, 1991). In cultured populations of *C. quadricarinatus,* various types of intersex individuals have been described, based on the observation of both male and female openings in the same individual (Medley and Rouse, 1993; Brummett and Alon, 1994). However, neither the sexual physiology

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Abbreviations: GSI = gonadosomatic index; EWI = endopod width index; AG = androgenic gland.

Table I

Frequency and morphometric description of sexual types in a 7-month-old population of Cherax quadricarinatus

Type	Total no. harvested	Sample size (*n*)	Body weight (g)	Carapace length (mm)	Propodus length (mm)	Red patch length (mm)
Male	531	50	53.1 ± 23.5^a	42.1 ± 6.0	48.1 ± 5.4^a	20.2 ± 19.9 (*n* = 46)
Female	514	50	43.3 ± 13.0^b	40.2 ± 4.2	42.1 ± 10.3^b	—
Intersex	13	13	41.9 ± 16.0^b	39.0 ± 5.3	43.2 ± 9.0^b	17.5 ± 7.5 (*n* = 12)

Values are means ± SD. Values sharing the same superscript letters are not significantly different.

of intersex crayfish nor the possibility of sequential hermaphroditism has been fully addressed.

To shed more light (from a different angle) on the development of maleness and femaleness in decapod crustaceans, we examined intersex individuals from a cultured population of *C. quadricarinatus* to determine the status of their reproductive system and the presence of androgenic glands. Morphological, anatomical, and biochemical characteristics were compared among male, female, and intersex crayfish. For the study of possible sequential sex changes, some intersex individuals were grown in the laboratory to 19 months of age.

Materials and Methods

Animals

Juvenile crayfish (*C. quadricarinatus*) were hatched at the Department of Aquaculture, Agricultural Research Organization, Bet-Dagan, Israel. At an average age of 1 month and a weight of 0.02–0.2 g, the juveniles were

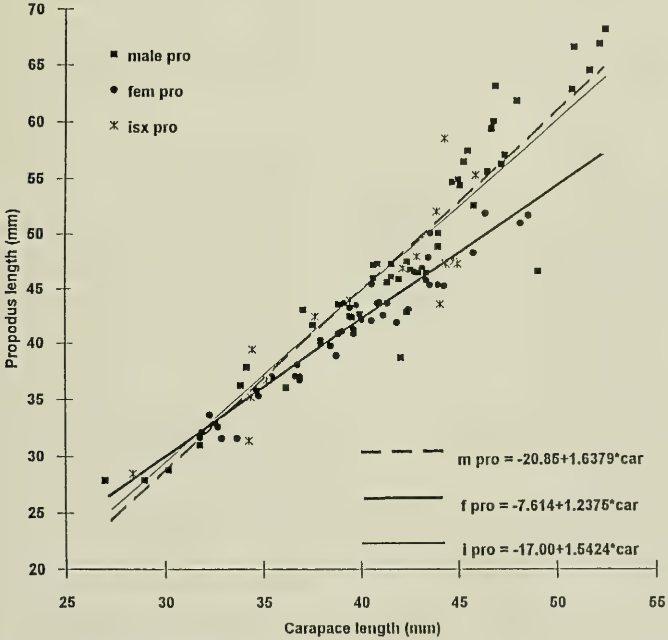


Figure 1. Regression line for propodus length *vs.* carapace length in 7-month-old intersex, male, and female *Cherax quadricarinatus*.

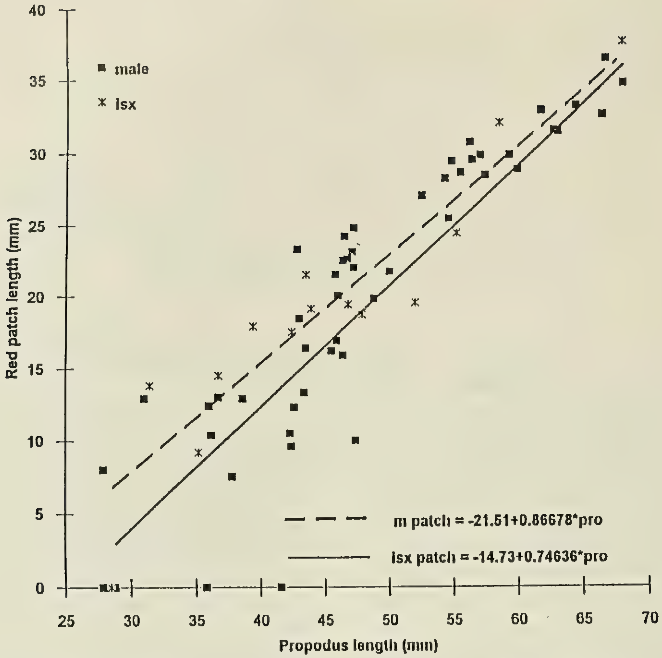


Figure 2. Regression line for red patch length *vs.* propodus length in 7-month-old intersex and male *Cherax quadricarinatus.*

moved to 400-m^2, 1-m-deep earthen ponds at the Dor Fish Culture Research Station. The crayfish were harvested after 6 months: 1058 individuals were examined externally, and their sex was determined according to the presence or absence of male or female genital openings. Individuals that had both male and female openings (total of 13) were classified as intersex. Six intersex individuals, harvested from a different population grown under similar conditions, were kept in aquaria at the Ben-Gurion University laboratory for an additional 12 months. These crayfish were fed daily with fresh ground fish flesh and vegetables, temperature in the aquaria was 27° ± 2°C, and water quality was maintained by recirculating the water through a gravel biofilter. At the age of 19 months, these animals were examined as described below.

Table II

Morphological properties of Cherax quadricarinatus *intersex individuals compared to males and immature and mature females*

	Sample size (*n*)	Red patch	EWI	Setation
Intersex	16	+	1.13 ± 0.07^a	Plumose
Male	6	+	1.17 ± 0.08^a	Plumose
Immature Female	8	−	1.30 ± 0.13^b	Plumose
Mature Female	9	−	1.95 ± 0.23^c	Simple and Plumose

EWI = endopod width index ± SD. Values sharing the same superscript letters are not significantly different according to Sime's multiple test.

Morphological observations

The following morphological variables were recorded at the time of harvest (age 7 months) for each intersex individual (n = 13), for random control samples of females and males (n = 50 of each sex) from the same population, and for the 19-month-old individuals (n = 6): body weight (±0.1 g), carapace length, propodus length (±0.1 mm). The presence and length (±0.1 mm) of a red cuticular patch along the propodus was recorded. This patch is a typical male secondary character (Thorne and Fielder, 1991) that elongates with maturation.

Along with the process of maturation in *C. quadricarinatus* females, changes in the morphology of the pleopod occur that facilitate its role in holding the newly deposited

Table III

Different combinations of male and female gonopores in Cherax quadricarinatus *intersex individuals and the anatomy of their reproductive systems*

Male gonopores	Female gonopores	*n*	Testis	Sperm duct	Ovary	Oviduct	Androgenic gland
Right	Right & left	10	Right	Right	Left	Left	Right
Left	Right & left	4	Left	Left	Right	Right	Left
Right	Left	1	Right	Right	Left	Left	Right
Right & left	Right	2	Right & left	Right & left	None	None	Right & left
Right & left	Left	1	Right & left	Right & left	None	None	Right & left
Right & left	Right & left	1	Right & left	Right & left	None	?*	Right & left

* On one side a rudimentary duct connecting the testicular tissue with the female genital opening was observed, in addition to the sperm duct.

eggs (Khalaila, unpub. data). The endopod of mature (vitellogenic) females is longer and wider than the exopod, and a mixture of plumose setae and long, thin simple (ovigerous) setae is present on the endopod. Pleopods were examined on all intersex individuals and on an additional sample of males (6), immature (pre-vitellogenic) females (8), and mature females (5) aged over 1 year. One of the third pair of pleopods was removed from each individual, and endopod and exopod width was measured with a pair of calipers to the nearest 0.1 mm. The relative growth of the two components of the pleopod, representing a female sex characteristic, was expressed as the endopod width index

(EWI = Endo. Width/Exo. Width).

The appearance of the setae (either plumose or a mixture of plumose and simple setae) attached to the endopod was studied under a light microscope.

Anatomical observations

To study the reproductive system, a sample of 11 (out of 19) intersex individuals of both age groups, 8 pre-vitellogenic females, 5 vitellogenic females (according to the pleopod features described above), and 6 mature males were dissected on ice. Their gonads, sperm ducts or oviducts, and androgenic glands were identified. The ovaries were removed and individually weighed after removal of excess water. The weights were used in calculating the gonadosomatic index

(GSI = (Ovary Weight/Body Weight) × 100).

A random sample of at least 15 fresh oocytes per ovary was placed under a light microscope and the oocyte diameter was measured with an objective micrometer (±10 μm). A sample of the contents removed from the subterminal region of the sperm duct was spread in saline on a glass slide and examined microscopically for the presence of mature spermatozoa.

Polypeptide profiles

Ovarian tissue was removed from 9 intersex individuals, 8 pre-vitellogenic females, and 5 vitellogenic females, homogenized individually on ice with 0.5 ml of 0.05 *M* Tris-HCl buffer, pH 7.2, and centrifuged at 10,800 × *g* for 15 min at 4°C. The amount of total protein in the supernatant was determined (Bradford, 1976). Samples (8 μg protein per lane) were separated by SDS-PAGE on 7% acrylamide gel (Laemmli, 1970) and stained with Coomassie blue.

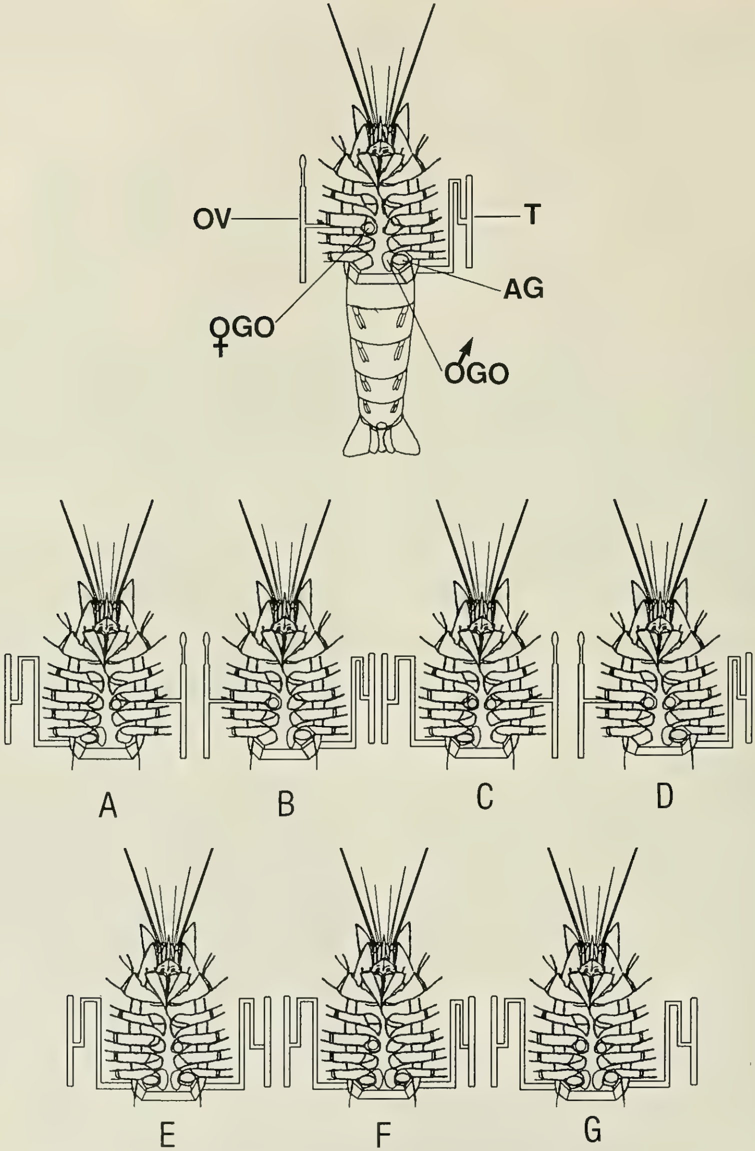
Statistical analyses

Data were analyzed using ANOVA, followed by Duncan's multiple range test, except for EWI and GSI. The latter are ratios, and their sample size was rather small, hence the Kruskal-Wallis nonparametric test was used for testing significance of difference among groups, followed by Simes's (1986) procedure for multiple tests. Probabilities below 0.05 and 0.01 were considered significant and highly significant, respectively.

Results

Morphology of intersex individuals

The population investigated—1058 crayfish grown at the Fish Culture Research Station—included 1.2% intersex individuals. All but one of the 13 intersex individuals developed cuticular red patches, a male sexual characteristic (Thorne and Fielder, 1991), on the propodus. Male crayfish were significantly heavier and had a longer propodus than did females and intersex individuals (Table I). No significant differences between males and intersex individuals were found in the carapace length or in the length of the cuticular red patch. The latter was missing in four males and one 7-month-old intersex individual. A highly significant regression of propodus length *vs.* carapace length was found for all three sex types ($r = 0.945$, 0.967, and 0.919 for males, females, and intersexes, respectively). The slope of the regression line for males was significantly different from that for females (Fig. 1). The slope of the regression line for intersexes was not significantly different from that for males or females (Fig. 1). The regression of the cuticular red patch length *vs.* propodus length was signif-


OV
T
AG
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♂GO
A
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C
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E
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Figure 3. Different types of *Cherax quadricarinatus* intersex individuals. T = testis; OV = ovary; GO = genital opening; AG = androgenic gland.

Table IV

Properties of the gonads and their products in Cherax quadricarinatus *intersex individuals compared to those of males, and pre-vitellogenic (P) and vitellogenic (V) females*

	Sample size (n)	Ovarian index (GSI)	Oocyte diameter (μm)	Sperm
Intersex	4	0.12 ± 0.05^a	292 ± 148^a	Present
Male	6	—	—	Present
Female (P)	8	0.42 ± 0.25^a	287 ± 87^a	Absent
Female (V)	5	2.15 ± 0.28^b	1020 ± 292^b	Absent

Statistical comparisons among groups were done by Simes's multiple test (for GSI) and Duncan's multiple range test for oocyte diameter. Values marked with the same superscript letters are not significantly different. Oocyte diameter represents mean of 15 oocytes per ovary.

icant in both male and intersex crayfish (r = 0.904 and 0.888, respectively), but the slopes of the lines were not significantly different (Fig. 2).

In intersex crayfish, the endopod of each pleopod was nearly equal in size to the exopod (EWI = 1.13 ± 0.07); a similar endopod/exopod ratio was found in males (EWI = 1.17 ± 0.08). In females the endopods were longer and wider than the exopods, and the EWIs of immature (1.30 ± 0.13) and mature (1.95 ± 0.23) females were significantly different from each other and from those of males and intersex individuals (Table II). In intersex male and immature female crayfish, each endopod and exopod had two distinct rows of delicate plumose setae. A mixture of plumose setae and long, thin simple setae was present in the endopod of mature females. All intersex individuals, from both age groups, possessed morphological properties, such as EWI and setation, that resembled the characteristics of males (Table II).

Male and female genital openings and the reproductive system of the intersex individuals

Seven combinations of genital-opening placement were found in the intersex crayfish (Table III). Anatomically, all dissected intersex individuals (from both age groups) that possessed a male opening also possessed a testis and a sperm duct on that side (Fig. 3). An androgenic gland was attached to the subterminal region of each sperm duct. Not all visible female openings indicated the presence of a female reproductive system, however. Individuals (from both age groups) in which a male and a female opening were present on one side possessed no ovary on that side (Fig. 3, C through G). In one case a rudimentary duct connecting the testicular tissue with a female genital opening was observed, in addition to the sperm duct. The identification of this duct was difficult because it was very thin and inconspicuous. An ovary with an oviduct was found only in cases in which a female opening was present in the absence of a male opening on the same side (Fig. 3, A through D).

Gonads and their products

The GSI of the ovarian component of intersex individuals (×2, since they possess only one ovarian lobe) (0.12 ± 0.05) was not statistically different from that of pre-vitellogenic females (0.42 ± 0.25) and was significantly smaller than that of vitellogenic females (2.15 ± 0.28) (Table IV). The diameter of the oocyte in the ovarian component of the intersex individuals (292 ± 148 μm) was similar to that of immature, pre-vitellogenic females (287 ± 87 μm) and significantly smaller than that of mature, vitellogenic females (1020 ± 292 μm, n = 4 ovaries) (Table IV). As in normal males, in intersex individuals the sperm duct contained viable spermatozoa.

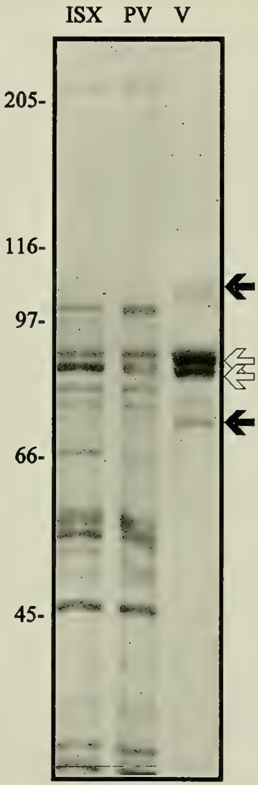

Figure 4. SDS-PAGE separation of polypeptides from a cytosolic extract of ovaries from a 19-month-old *Cherax quadricarinatus* intersex (ISX) compared to a pre-vitellogenic female (PV) and a vitellogenic female (V). ⇦ = Polypeptides present in all lanes. ⬅ = Polypeptides present only in the vitellogenic ovary.

The polypeptide profile of the cytosolic component of the ovary of intersex individuals (from both age groups) was similar to that of pre-vitellogenic female ovary (Fig. 4: lanes 1 and 2, representing an intersex of 19 months and a pre-vitellogenic female, respectively). The Coomassie-blue-stained polypeptides with molecular weights of about 204, 199, 106, 85, 59, and 43 kDa were the most prominent polypeptides in both the intersex ovary and the female pre-vitellogenic ovary. These polypeptides were not noted in the vitellogenic ovary. The polypeptides with molecular weights of about 93 and 90 kDa were present and pronounced in all three types of ovary (lanes 1, 2, and 3, Fig. 4, white arrows). The most prominent polypeptides in the vitellogenic ovary had molecular weights of about 111 and 76 kDa (lane 3, Fig. 4, dark arrows). These polypeptides were present in the vitellogenic ovary and were detected neither in the pre-vitellogenic female ovary nor in the ovary of intersex individuals.

Discussion

Intersexuality is the occurrence in a protandric, protogynic, or normally gonochoristic species of an individual with both male and female characteristics. These characteristics may be limited to the external morphology or they may extend to gonadal differentiation. The frequency of intersex individuals in cultured populations of *C. quadricarinatus* has been reported to range from 2%–4% (Brummett and Alon, 1994) and from 4% (Thorne and Fielder, 1991) up to 17% (Medley and Rouse, 1993). The 1.2% frequency of intersex individuals presented in this study is relatively low. This frequency was consistent for the same breeding population over several years (unpub. data): it was 1.3% in 1993 (Karplus *et al.,* 1995). Recently, based on external observations, Medley and Rouse (1993) described five types of intersex individuals in cultured populations of *C. quadricarinatus,* including one animal with a complete testis on one side and what appeared to be undeveloped ovarian tissue on the other side. Our results confirm these observations in that they show that intersex *C. quadricarinatus* individuals that have a male genital opening also have a testis, a sperm duct, and an androgenic gland on that side (Fig. 3). On the other hand, we observed an ovary only in cases in which a female genital opening was present in the absence of a male genital opening on the same side (Fig. 3, A through D).

Medley *et al.* (1994) observed a normal testis and a pre-vitellogenic ovary in a single *C. quadricarinatus* intersex individual, and described it on the basis of histological examinations as a case of "true hermaphroditism." They reported one intersex individual that had a right-side male genital opening and two female genital openings and functioned as a male, siring a batch of offspring. Our results agree with the observation that intersex *C. quadricarinatus* (from both age groups) are functional males. Indeed, all but one of the intersex individuals examined developed the red cuticular patch (a male secondary sexual characteristic), but none developed ovigerous simple setae (a female sexual characteristic). True hermaphroditism, in which both male and female reproductive systems are functional, either simultaneously or sequentially, was not observed in the present study. Clarification of the function of the female component of the reproductive system in intersex crayfish requires further investigation. The intersex phenomenon seems to be a stable state rather than a transient one since no changes occurred in intersex individuals monitored from 7 up to 19 months of age.

With respect to the role of the androgenic gland (AG) in regulating crustacean sex differentiation, Nagamine and Knight (1987a) stated that "knowing that the AG can masculinize genotypic females makes the presence of a bilateral gynandromorph a paradox since the AG at the male half should be capable of masculinizing the contralateral female half." The authors suggest an explanation in which the feminized half of the bilateral gynandromorph consists of "AGH receptor-minus cells." That explanation may apply to the presence of bilateral gynandromorphs such as those described in the Bay prawn *Nephrops norvegicus* (Farmer, 1972). In this species, an individual possessing both male and female genital openings was described as a rare case (1:40,000), presenting a complete bilateral asymmetry in which one side had male primary and secondary sex characters while in the other half only female characters were expressed. The expression of intersexuality in *C. quadricarinatus,* in which the secondary external characters were masculine on both sides (red patch, pleopod morphology, etc.), may call for a different explanation. It better conforms with the hypothesis that male differentiation in decapods is mediated by a substance that is secreted from the androgenic gland primordia and diffuses along the genital tract (Charniaux-Cotton and Payen, 1988). This may explain the presence of a male reproductive system and the absence of a female system on the same side, *i.e.,* the side on which the androgenic gland exerts its local effect through diffusion; on the other side, in the absence of an androgenic gland, differentiation of an ovary is permitted (Charniaux-Cotton, 1959).

Our findings showed that intersex individuals of both age groups possessed an androgenic gland and an active testis, but the ovarian component was always pre-vitellogenic. The latter observation was confirmed by the polypeptide profile of the intersex ovarian component; the profile was similar to that of an ovary from a pre-vitellogenic female and did not contain the specific polypeptides typical of the vitellogenic ovary (Fig. 4). These findings are in agreement with reports in the literature that oogenesis can proceed in the testes of hermaphrodites until the end of the primary vitellogenesis (Charniaux-Cotton, 1965; Charniaux-Cotton and Payen, 1985). Inhibition of vitellogenesis by androgenic hormones has also been reported (Berreur-Bonnenfant and Lawrence, 1984). The authors of that study suggest that the androgenic gland exerts an effect on remote target organs, via the circulation, in addition to its effect, by local diffusion, on the differentiation process.

Mature *C. quadricarinatus* females possessed vitellogenic ovaries and ovigerous setae; intersex individuals and immature *C. quadricarinatus* females possessed pre-vitellogenic ovaries but did not have ovigerous setae. The development of ovigerous setae in crustaceans is regulated by an ovarian factor (Nagamine and Knight, 1987b). We suggest that this factor is not synthesized by the ovaries of intersex individuals and immature females. Suzuki and Yamasaki (1991) found that female secondary sex characteristics were induced by the presence of vitellogenic ovaries, whereas pre-vitellogenic ovaries had no effect. The absence of ovigerous setae in intersex individuals (possessing an androgenic gland) may be explained by the antagonistic effect of the androgenic hormone on the ovarian factor (Suzuki and Yamasaki, 1991).

In these respects, intersex *C. quadricarinatus* individuals present a unique model for the study of the various regulatory roles of androgenic hormones and ovarian factors in sex differentiation and gonadal function in decapod crustaceans.

Acknowledgments

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Completely Direct Development of *Abatus cordatus*, a Brooding Schizasterid (Echinodermata: Echinoidea) from Kerguelen, With Description of Perigastrulation, a Hypothetical New Mode of Gastrulation

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Abstract. Development of the brooding schizasterid *Abatus cordatus,* a subantarctic echinoid endemic to Kerguelen, is described. Females spawn nonbuoyant eggs 1300 μm in diameter, which are fertilized by elongated sperm (head 1 μm wide and 15 μm long). The main characteristics of this development are (1) incomplete cleavage beginning at the animal pole that becomes holoblastic, giving a filled wrinkled blastula 26 days after fertilization; (2) apparent (fate-mapping studies have not been done) external migration of mesenchyme cells, in the perivitelline space, from the animal to the vegetal pole during gastrulation while the archenteron invaginates; (3) hatching occurring at the end of the gastrulation (65 days after fertilization); (4) differentiation of the vestibule from a thickening of the oral epidermis as soon as the end of gastrulation is attained; and (5) production of a juvenile directly from the gastrula without any larval stage. The juvenile that leaves the brood chamber is 2 mm in diameter and about 250 days old. *A. cordatus* is a true completely direct developer (no larva and no metamorphosis). We propose to use (1) the term *perigastrulation,* as a tentative one until more definitive studies are available, to describe the hypothetical peculiar movement of cells during gastrulation and (2) the terms of *direct development* only for completely direct developing species and *abbreviated development* for species that have more or less transformed plutei.

Introduction

A great diversity of larval forms and patterns of development occur in echinoderms. They are often classified on characters such as larval and embryonic features, habitat, and nutritional type (*cf.* McEdward and Janies, 1993). Raff (1987) considered four patterns of development in echinoids for the transition from the classical feeding, free-swimming larva echinopluteus to completely direct development. However, in asteroids, McEdward and Janies (1993) distinguished eight possible patterns on the basis of three characters (development, habitat, nutrition) each with two states (respectively, indirect or direct, pelagic or benthic, feeding and nonfeeding). In fact, such a multifactorial classification, which encompasses all of the possible combinations, is also valid for echinoids. From a strictly embryological point of view, development is described following the distinction between direct and indirect. A general definition of indirect development, and of larva and metamorphosis, has been proposed by Geigy and Portmann (1941). According to them, a larva goes through a metamorphosis that is characterized by three processes: (1) obliteration of the larval parts of the animal, (2) formation of certain adult parts from rudiments that remained in an embryonic state, and (3) continuation of larvo-adult formations that are not affected by metamorphosis. This refers to a pattern of development in which the gastrula is followed by intermediate stages with structural features that are not directly involved in the morphogenesis of the juvenile (McEdward and Janies, 1993). These authors defined direct development as a pattern wherein the embryonic stages are followed by the morphogenesis of the juvenile, without an intervening larval stage. Considering modified developments, the problem is, what is a larva? McEdward and Janies (1993) proposed that "a larva is produced by post-embryonic morphogenesis (postgastrula) and is eliminated by the metamorphic

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transition to the juvenile." Thus, considering the evolutionary diversification of development, to assume a simple dichotomy between direct and indirect development is very difficult and appears to be an oversimplification. Indeed, when nutritional and swimming larval structures are reduced or even absent, development is called abbreviated (Strathmann, 1978; Emlet *et al.,* 1987; Raff, 1987; Pearse *et al.,* 1991; Amemiya and Emlet, 1992). Such a decrease in morphological larval complexity led some authors to arrange larvae along a continuum reaching from typical planktotrophy to the absence of any larval stage (direct development) and to the corresponding internal reorganization of the embryo (Raff, 1987; Raff *et al.,* 1990; Olson *et al.,* 1993). For these authors, other changes that occur in the shift to direct development are the increase of egg size and an acceleration in the appearance of juvenile features. However, even if series of reductions in larval structures have been described in some lineages, such series are not evident in most groups of echinoderms (Emlet *et al.,* 1987). If a continuun does exist, it has been achieved through time. Thus the "terminal" observation of present larval types may give only an approximate idea of the process.

Further detailed descriptions of the developmental patterns are needed to fully understand mechanisms underlying evolutionary changes (Raff, 1992). Information on the internal morphological events occurring during modified development is limited (see Barker, 1985; Amemiya and Emlet, 1992; Parks *et al.,* 1989), and data on echinoid species with completely direct development are scarce. Brooding rarely proceeds by strictly direct development. Benthic brooding may involve more or less simplified larval stages (McEdward and Janies, 1993). Of the perhaps 10 to 30 species with modified development (Emlet, 1990), sometimes qualified as direct (see Raff *et al.,* 1990; Olson *et al.,* 1993), only *Goniocidaris umbulacrum* (Barker, 1985, peri-oral brooder) seems to have a completely direct development, with no larval feeding appendages, no necrosis of larval tissue, and no metamorphosis (ametamorphic development [Bonar, 1978]). *Phyllacanthus parvispinus,* another cidarid echinoid, may be another directly developing species (Parks *et al.,* 1989). More recently, *Holopneustes purpurescens* has been described as an apluteal developing species (Morris, *in press*).

The present article elaborates on previous information about the events occurring from spawning to settling in *Abatus cordatus,* an echinoid with completely direct development (Schatt 1985a, b; 1988; Schatt and De Vos, 1990). The aims of the article are to give (1) an overview of the true direct development of this brood-protecting spatangoid that is endemic to the Kerguelen Islands (South Indian Ocean, 70°12'E, 49°21'S) and (2) preliminary evidence for a new mode of gastrulation. The ecology and biology of this species are already well known (Magniez, 1980, 1983; Schatt, 1985a; Poulin and Féral, 1994, 1995) and are similar to those of other *Abatus* spp. (Pearse and McClintock, 1990). Reproductive (Magniez, 1980) and brooding cycles are annual in Kerguelen and may depend on the locality and the depth where the animals live (Schatt and Féral, 1991); it is therefore possible to obtain and study all developmental stages.

Materials and Methods

Collection and preparation of Abatus cordatus

Female *Abatus cordatus* (Verrill, 1876) were collected fortnightly from December 1981 to March 1983 in the fine sand of the Anse du Halage in the Golfe du Morbihan. Samples collected in 1986, 1988, 1990, and 1992 were also used to check some details.

Females were held in the laboratory (BIOMAR, Base of Port-aux-Français) before analysis. Brooded young were collected from each of the four pouches of the female, sorted under a binocular microscope, and reared in glass vessels containing filtered seawater (Millipore 0.45-μg filter) which was renewed every 2 days. They were maintained in the dark, from 7°C (in summer) to 2°C (in winter). No food was added, since internal nutrient stores and, possibly, dissolved organic matter [DOM] (Féral, 1985; Walker and Smith, 1985) supported development through the advanced juvenile stage. Artificial fertilization was performed in order to observe the first cleavage. Oocytes were collected directly in the gonoduct. They were fertilized by sperm ($\sim$250,000 cells $\cdot$ ml^{-1}) in filtered seawater.

Preparation for light microscopy and scanning electron microscopy

Developmental stages were fixed in 3% glutaraldehyde with 0.2 M sodium cacodylate, at pH 7.4 (final concentrations, 1100–1200 mOsM). Serial semi-thin sections (1 μm thick) were cut after embedding in araldite. They were stained with toluidine blue (pH 11.5).

For scanning electron microscopy (SEM), developmental stages were fixed in the same mixture and postfixed with 2% osmium tetroxide in the same buffer. Embryos and juveniles were dehydrated in graded ethanol and dried by the critical-point method. They were mounted on aluminum stubs, coated with gold in a sputter coater, and observed with an ISI DS 130 scanning electron microscope.

For DNA staining, eggs were fixed in buffered 10% paraformaldehyde, then rinsed in 150 mM NaCl, 50 mM TRIS, pH 7.5, containing 0.1 μg $\cdot$ ml^{-1} Hoechst 33258.

Results

Gametes, spawning, and fertilization

The spherical egg is 1300 μm in diameter and is negatively buoyant. It is deep orange, densely yolked, and

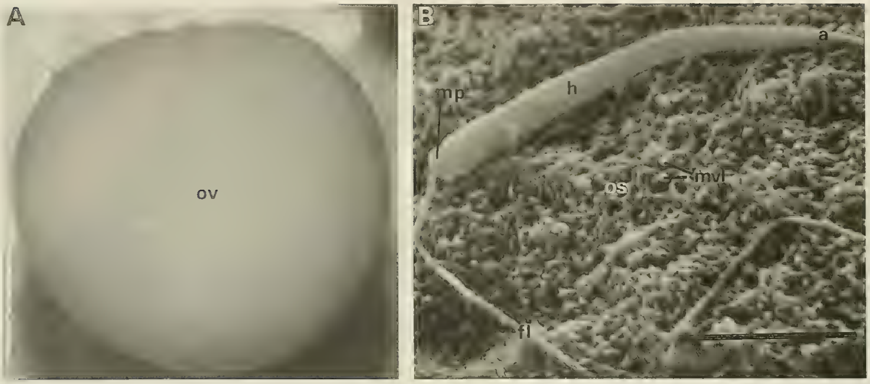


Figure 1. Female and male gametes of *Abatus cordatus.* (A) Ripe oocyte (light micrograph). (B) Spermatozoid (SEM micrograph). Abbreviations: a, acrosome; fl, flagellum, h, head; mlv, microvilli; os, oocyte surface; mp, middlepiece. Scale bar = A, 500 μm; B, 5 μm.

opaque (Fig. 1A). The sperm has a narrow, pointed head, 15 μm long and 1 μm wide, with a tail 25 μm long (Fig. 1B). During the breeding season, just after spawning, the eggs are moved to the brood pouches (Fig. 2A) by the spines and pedicellaria. Fertilization probably occurs during the movement of the eggs from the gonopore to the pouches. The fertilization membrane and a fertilization cone (Fig. 2B) lift off from the surface of the egg after about 34 min at 6°C, leaving a large perivitelline space of 65 μm. *In vitro,* the fertilization cone is always oriented to the bottom. The axis of polarity is approximately the axis of gravity and will be the same until the post-gastrular stage. The cleavage will affect the pole opposite the site of sperm entry, which is defined as the vegetal pole (facing downward). The fertilization membrane is present to the end of gastrulation.

Development before hatching

Cleavage and blastulation. A horizontal constriction, whose plan is perpendicular to the animal–vegetal axis, occurs 2 days after fertilization (Fig. 3A). It separates the egg into two parts, with a nucleus in the animal part and a non-nucleated vegetal part filled with yolk only. The second cleavage division (Fig. 3B), perpendicular to the constriction and involving only the animal part, forms two blastomeres. The blastomeres are yolky, ovoid to spherical, and 100 to 200 μm in diameter (Fig. 3C). Synchrony of division is lost as early as the third cleavage.

The vegetal yolk mass progressively decreases in size while the blastomeres increase in number (Fig. 2D and 4A). The yolk mass was not observed to be taken up by blastomeres (Fig. 3C). The yolk mass disappears and segmentation finally becomes total (Fig. 4B and 4C). The space between the blastomeres communicates with the perivitelline space (Fig. 4C). A blastocoele was never observed. The blastula is filled with cells that originate from the blastomeres. The cell nucleus is rarely visible because of the abundance of yolk; moreover, the large size of the mitotic apparatus (almost 100 μm, Fig. 3B) obstructs the nuclear area in 1- to 2-μm-thick sections. On semi-thin sections, often only clearer areas ("ghosts" of nuclei) can be seen in some cells (Fig. 4C). All the cells of the blastula are filled with lipid(?)-rich spheroids.

Eighteen days after fertilization, the blastomeres associate into lobes, giving the blastula a wrinkled appearance. The blastomeres become smaller and smaller through divisions, thus increasing the ratio of surface area to volume. The wrinkling may be due to a lack of space in the tight-fitting fertilization membrane. The embryo is convoluted by furrows that gradually spread over its lumpy surface. About 26 days after fertilization, the stereoblastula is totally wrinkled (Figs. 2E and 4D) and consists of a monostratified columnar epithelium enclosing a mesenchyme mass.

Gastrulation. Within a few days, the wrinkled blastula transforms into a young, externally smooth gastrula (Fig. 2F). It will develop to hatching in the fertilization membrane that always keeps the same overall size (1.3 mm in diameter). A large depression, corresponding to the blastopore, appears at the vegetal pole 30–35 days after fertilization. The gastrula consists of a central mesenchyme surrounded by a monolayered epithelium, whose cells are cylindrical, still yolk-rich, and 100 ± 10 μm in height. The vegetal part of this gastrular epithelium invaginates to form the archenteron (Fig. 5D), while the animal part of the epithelium becomes thinner (Fig. 5B and E). The early gastrula is filled with yolky mesenchyme cells (Fig.

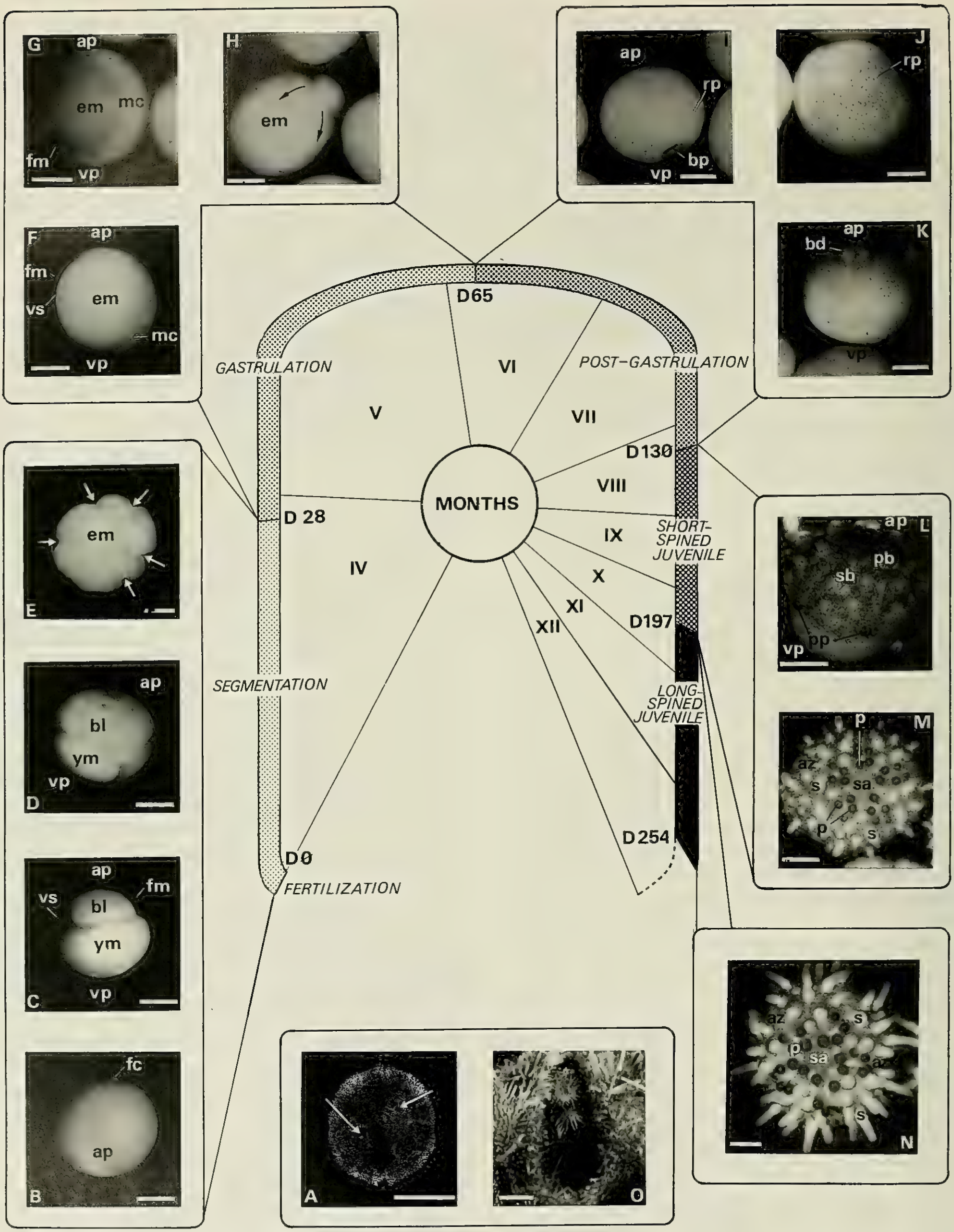
5D) in each of which the nucleus is difficult to see because of the very abundant yolk (Fig. 5E). As early as the onset of invagination, cells emerge from the thinner part of the epithelium and enter the perivitelline space at the animal pole (Fig. 5B). Their microanatomy is similar to that of the mesenchyme, suggesting that they originate from it, through the ectoderm (Fig. 5B). At the animal pole, the gastrular epithelium may become locally disorganized (Fig. 5E). During gastrulation, these mesenchyme-like cells move around the embryo, from the animal pole to the deepened blastopore in the perivitelline space, forming a lateral cap on half of the surface of the embryo (Figs. 2F and 6A). More and more cells invade the perivitelline space as the diameter of the gastrula decreases (Figs. 2G and 6B). Finally, the perivitelline space is again completely free of cells (Fig. 6C). In fact, all the migrating cells have moved to the blastopore of the embryo to form, at the end of gastrulation, a "vitelline plug" (Fig. 6C) that closed and thus isolated the archenteron in the mesenchyme (Fig. 7C). All that remains visible of the mass of migrating cells at the surface of the embryo is the vitelline plug at the blastopore. During gastrulation, the lumen of the archenteron is always empty. The advanced gastrula is still filled with mesenchyme cells (Fig. 7C), and interphase and mitotic nuclei are often observed in the mesenchyme (Fig. 7D). At the end of gastrulation (50–65 days), part of the animal ectoderm thickens and forms two tissue layers separated by a lumen. This cell mass will become the vestibule (Fig. 5C). The external layer becomes the ceiling of the vestibule. The internal layer (floor of the vestibule) and the underlying mesenchyme will give rise to the oral surface of the juvenile. Other cell masses differentiate within the mesenchyme (Fig. 7A and B). They grow hollow and give rise to the coelomic cavities. No connection between these cell masses and the archenteron was ever observed on serial sections. Gastrulation ends with hatching (65 days after fertilization). The embryo breaks through the enclosing fertilization membrane (Figs. 2H and 6D).

Post-gastrular stage and differentiation of the oral surface of juvenile

The embryo is now spherical (1.3 mm in diameter). A small aboral depression is the only mark of the blastopore (Fig. 2I). During the post-gastrular stage, from 65 days to about 130 days after fertilization, a red pigmentation spreads over the surface of the postgastrula embryo and finally concentrates at the oral pole (Fig. 2J). It is surrounded with an epidermis 70 to 80 μm thick (Fig. 8A and C). The developing vestibule, arising from the ectodermal cell mass, is now separated from the embryonic epidermis (Fig 8A) and completely surrounded by mesenchyme (Fig. 8A and B). The coelomic cavities differentiated from the mesenchymal cell masses of the gastrula increase in volume around the gut (Figs. 8C and 9A). The gut wall consists of three tissue layers: a digestive epithelium (high and narrow cylindric cells filled with yolk), an intermediate layer of mesenchyme, and a thin coelomic epithelium (Fig. 8B, C). At the end of the post-gastrular stage, the digestive tube is suspended in the coelomic cavity by means of mesenteries (Figs. 8C and 9A). The future oral surface of juvenile has formed from the ectodermic floor of the vestibule and the underlying mesoderm (Figs. 8B, C, and 9E). It is separated from the external environment by the epidermis of the postgastrula embryo and the epidermis of the ceiling of the vestibule (Figs. 8C, 9A and E). The epidermis of the floor of the vestibule forms five external folds that will become the five primary podia (Figs. 8B, 9B and C). Internal folds of that floor will give rise to the neural plate (Fig. 9A and D) and the five radial nerves (Fig. 9B). These folds are epineural folds that are growing over the juvenile oral surface to form the epineural sinus, as in the juvenile rudiment of planktotrophic larvae (von Ubisch, 1913; Hyman, 1955; Emlet, 1988). At the same time, the underlying mesoderm begins to differentiate the aquiferous ring, which is extended under each fold to form five aquiferous tubes (Fig. 9B and D). The radial nerves and the aquiferous tubes provide the primary organization of the ambulacral zone of the oral surface of the juvenile. The epidermis of the oral surface develops more buds; these will evolve into additional podia in the ambulacral zone and into spines (Fig. 9C and E). Between 80 and 85 days after fertilization, a tiny fold of the ectoderm ceiling of the vestibule forms and grows toward the embryonic epidermis (Fig. 8D). Fifteen days later, the epidermal ceiling of the vestibule merges into the oral embryonic epidermis (Fig. 8F), forming a tiny opening to the external environment (Figs. 2K, 8E and F). At the end of the post-gastrular stage, the epidermis of the oral (animal) pole becomes thinner and thinner until it turns almost transparent (Fig. 8E). Finally, this opening becomes bigger and bigger. The lips move to the aboral (vegetal) pole. The movement involves the total exteriorization of the oral surface of juvenile. At this stage (130 days), the "old" postgastrula embryo measures about 1.6 mm in diameter. That means a growth of 0.3 mm in diameter. Classical echinoid larval structures (arms, ciliary bands, larval tissue, or larval skeleton) have never been observed during the post-gastrular stage.

Juvenile stages

The brooding cycle continues with two juvenile stages. Externally, these two stages differ markedly in spine length (Magniez, 1979): stage 1 (130–197 days) has short spines (Fig. 2M); stage 2 (197–254 days) has much longer spines (Fig. 2N). Both are orange with much red pigment in the epidermis. Except for the gut, all organs of the juveniles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essentially originate from the differentiation of the floor of the oral surface of the juvenile.

Short-spined juvenile (juvenile stage 1). The early juvenile stage (Fig. 2L) is characterized by a complete exteriorization of the stomodeal area and of the five ambulacral areas that end in primary podia. They will extend to the aboral pole during this phase. Juvenile stage 1 is oblong in outline but becomes spherical (1.8 mm in diameter) as development proceeds.

At the oral pole, the coelomic cavity increases in volume and progresses to the aboral pole. The gut is suspended in the coelomic cavity by means of mesenteries. The digestive tube convolutes on itself, forming two loops as revealed by serial sections (see Fig. 10B). The upper loop is the most developed and goes counterclockwise; the smaller, second one goes clockwise. The ends of the gut are still not differentiated. Although the oral end is virtually in its final position, near the stomodeal area (Fig. 10B), it is not yet connected to the ectoderm (Fig. 10C). The latter begins to differentiate the stomodeum at the oral pole (Fig. 10C). The other end of the gut is in contact with the remaining mesenchyme (Fig. 10B), where the aboral mesentery differentiates. The mesenchyme is still filling the vegetal half of the juvenile (Fig. 10A, B). The three tissue layers forming the gut wall are very similar to that of the postgastrula embryo. The nervous system is made of the neural plate (Fig. 10C) and the five radial nerves (internal folds), which reach to the proximity of the primary podia (external folds). The aquiferous system begins to differentiate podial anlagen. Its gross anatomy is similar to that of the postgastrula embryo.

The advanced stage differentiates many small, nonmobile spines of *ca.* 0.1–0.3 mm in height (Fig. 2M) with nonfunctional tubercles and podia buds. The proctodeal area is now differentiated. It is surrounded by the five primary podia and is still closed by the ectoderm (as is the stomodeal area).

The very first skeletal stereom elements are now visible in the oral mesoderm, mainly in the interambulacral areas at the oral pole.

Long-spined juvenile (juvenile stage 2). The body of juvenile stage 2 is covered with many long, movable spines. It is spherical, about 1.95 mm in diameter without the spines. The epidermis includes many pigment cells, especially in the podia and at the bases of the spines (Fig. 2N, 11A). Both are pigmented deep red. During this phase the remaining mesenchyme cells have completely disappeared (Figs. 11B and C, 12A). Ectodermic invaginations of the stomodeal and the proctodeal areas join the digestive tube, but it remains closed (Fig. 12B). The mouth and the anus (Fig. 12E) will open only 1 week before the release of the juvenile (245–250 days after fertilization).

The digestive tube is now complete (Figs. 11B and C, 12A). Differentiation of the endoderm into digestive epithelium begins from the ends toward the middle part of the gut. This epithelium includes secretory cells and other cell types with well-developed microvilli (Fig. 12E). The coelomic epithelium consists of flagellated choanocyte-like cells. The hemal system and the siphon differentiate in the connective tissue (Fig. 11C). Digestive annexes (expansions of the stomach and the intestinal caecum) are also formed (Fig. 11C).

The oral area now includes a nerve ring, an aquiferous ring, and a hemal ring, which surround the future mouth (Fig. 12B). Five ambulacral canals, which originate from the stomodeal rings, consist of a nerve tube and an aquiferous tube and extend to the proctodeal area (Fig. 12C). The nerve ring and tube consist of an epineural sinus and three tissue layers: the epineural, ectoneural, and hyponeural systems (Fig. 12C). One podium (external expansion) and its ampulla (internal expansion) protude from the aquiferous canal alternately on each side along the radial ambulacra (Fig. 12C and D). They form the ambulacral area (Figs. 2N, 11A). Spines and buds of pedicellaria develop in the interambulacral area. Spines of the

Figure 2. Light micrographs of developmental stages of *Abatus cordatus* (Reference to the brooding cycle of Anse du Halage [about 250 days], Kerguelen Islands). (A) Female with one of the four brood pouches opened by discarding the protective spines. Arrows show embryos. (B) Oocyte 1 h after fertilization (the vegetal pole is turned up). (C) First [vertical] cleavage (2 days after fertilization) that gives the two first blastomeres upon horizontal constriction that separates the yolk mass (D) Young morula (8 days old). (E) Wrinkled stereoblastula (26 days old). Arrows indicate furrows. (F) Young gastrula (32 days old). (G) More advanced gastrula. (H) Hatching of the gastrula giving birth to the embryo. Arrows show the breaking of the fertilization membrane. (I) Young post-gastrula embryo. (J) Fully pigmented post-gastrula embryo. (K) Late post-gastrula embryo. The oral surface of the future juvenile is visible under the oral epithelium that is opening. (L) Young, short-spined juvenile (J_1). (M) More advanced, short-spined juvenile viewed from the oral pole. (N) 240-day-old long-spined juvenile (J_2) viewed from the oral pole. (O) Late, long-spined juveniles in place in a brooding pouch just before "birth." Abbreviations: ap, animal pole; az, ambulacral zone; bd, spine or podia bud; bl, blastomere; bp, closed blastopore; em, embryo; fc, fertilization cone; fm, fertilization membrane; mc, migrating cells; p, podia; pb, podia bud; pp, primary podia; rp, red pigmentation; s, spine; sa, stomodeal area; sb, spine bud; vp, vegetal pole; vs, vitelline space; ym, yolk mass. Scale bar = A, 2 cm; B to M, 500 μm; N, 1 mm; O, 250 μm. Time scale is logarithmic. Roman numerals in the center refer to calendar months.

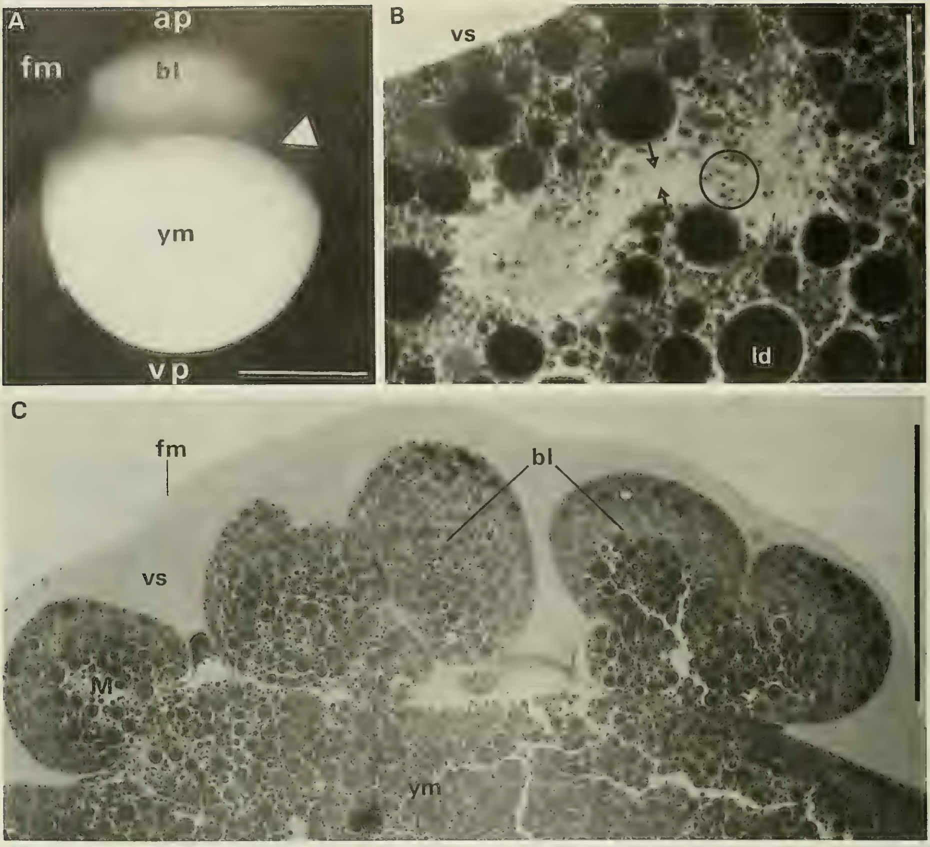

Figure 3. Segmentation of *Abatus cordatus.* (A) Constriction (arrow), 2 days after fertilization. (B) Second mitosis (anaphase) of one of the two first blastomeres (4 days after fertilization). Arrows show mitotic spindle, condensed chromosomes are visible in the open circle. (C) Section through the blastomeres at the animal pole (6 days after fertilization). They are all separated from the yolk mass. A is a videotape frame, B and C are semi-thin plastic sections (toluidine blue, pH 11.5). Abbreviations: ap, animal pole; bl, blastomere; fm, fertilization membrane; M, mitosis; vp, vegetal pole; vs, vitelline space; ym, yolk mass. Scale bar = A, 500 μm; B, 50 μm; C, 250 μm.

advanced juvenile stage 2 have a functional tubercle (muscles, catch apparatus, and skeleton) and are 0.5 to 1.2 mm in height. Juvenile stage 2 individuals are able to move, even in the pouches. The skeleton differentiates from the oral area, in the connective tissue toward the aboral area. When the broods are released from the pouches, a complete test is built by anastomosing perforated plates (Fig. 12D).

Discussion

Compared to other echinoids that produce a feeding larva or develop from a large egg through an abbreviated development, the most obvious peculiarity of *Abatus cordatus* is the lack of any larval stage and the tremendous increase in the length of its direct development. The juveniles become free-living 8.5 to 9 months after fertilization. The main characteristics of this development are (1) incomplete cleavage that becomes holoblastic; (2) external migration of mesenchyme cells, in the perivitelline space, from the animal to the vegetal pole during gastrulation; (3) hatching that occurs at the end of the gastrulation; (4) differentiation of the vestibule as soon as the end of gastrulation is attained; and (5) production of a juvenile directly from the gastrula with-

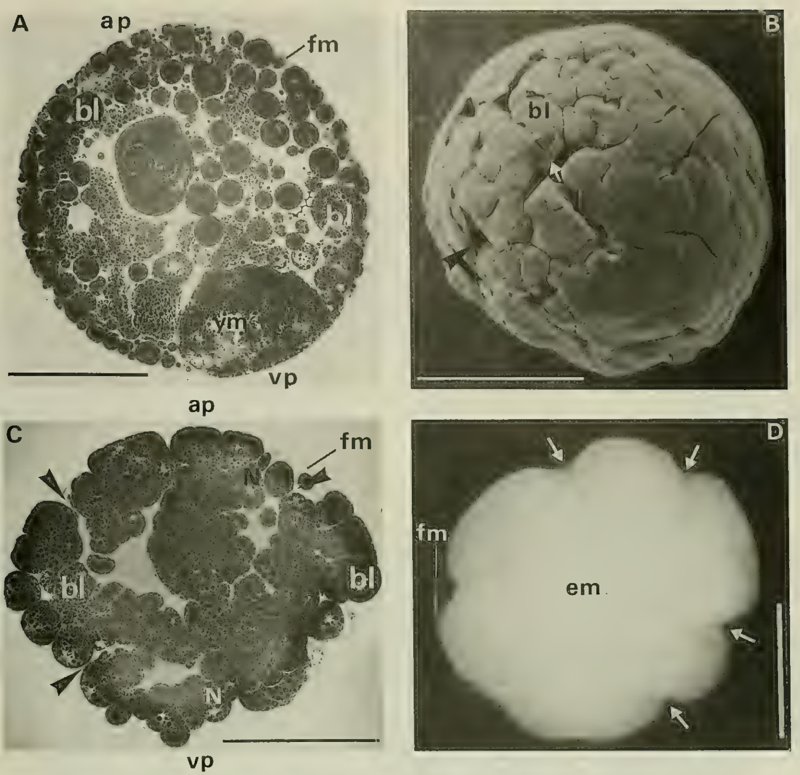

Figure 4. Segmentation of *Abatus cordatus.* (A) Section through a cleaved egg showing blastomeres and the remaining yolk mass at the vegetal pole (11 days after fertilization). (B) SEM view of a completely cleaved egg (14 days after fertilization). Blastomeres are visible where the fertilization membrane is destroyed (white arrow). Black arrow shows a furrow. (C) Section through a cleaved egg at the end of the segmentation (15 days after fertilization). Black arrows show the large intercellular spaces that communicate with the perivitelline space at the level of the furrows. (D) Light micrograph of a wrinkled stereoblastula (26 days after fertilization). Arrows show furrows. A and C are semi-thin plastic sections (toluidine blue, pH 11.5). Abbreviations: ap, animal pole; bl, blastomere; em, embryo; fm, fertilization membrane; N, nuclear area; vp, vegetal pole; ym, yolk mass. Scale bar = 500 μm.

out any larval stage. We will discuss some features of this completely direct development.

Large gametes and lecithotrophy

Chia *et al.* (1975) described the possible implications of sperm morphology for mode of fertilization and phylogeny. Raff *et al.* (1990) proposed a correlation between sperm morphology and developmental mode and phylogeny. Among nonfeeding developers, *Abatus cordatus,* with a 15-μm-long spermatozoid head and an egg 1300 μm in diameter, has the longest echinoid sperm after *Phyllacanthus parvispinus* (20 μm; Raff *et al.,* 1990), whose egg is not as large (700 μm in diameter; Mortensen, 1921). Moreover, the egg of *Araeosoma fenestratum* (1290 μm in diameter; Eckelbarger *et al.,* 1989a) corresponds to a sperm-head length of 8.5 μm. *A. cordatus* is near the upper limit of the range of sperm-head length for nonfeeding developers (6.3 to 20 μm, Raff *et al.,* 1990). These data show that spermatozoid length is only roughly correlated with mode of development. The mean length (±95% confidence interval), calculated from the compilation of Raff *et al.* (1990) and including *A. cordatus,* is 10.6 ± 7.2 μm. This indicates that an elongate-headed spermatozoid is consistent with a greater ability to penetrate a very large egg, with or without a thick jelly coat,

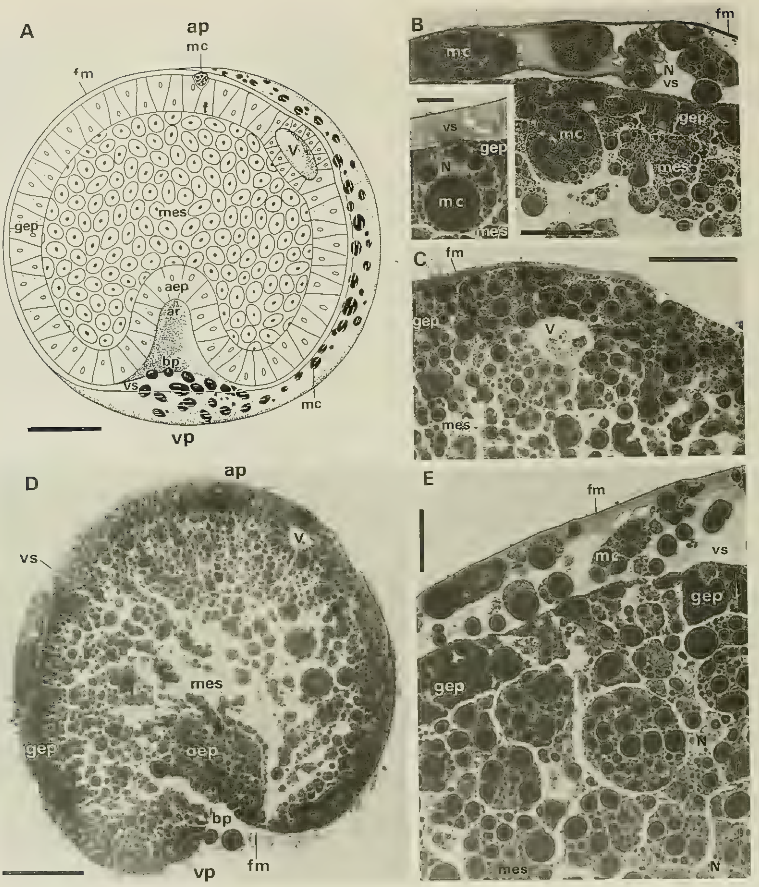

Figure 5. Gastrulation of *Abatus cordatus.* (A) Condensed representation of gastrulation. The vestibule is differentiated only in the late gastrula. (B) Broadcast of the mesenchyme cells through the gastrular epithelium from the mesenchyme to the vitelline space at the animal pole. Inset shows an infiltrated nucleated mesenchyme cell in the gastrular epithelium. (C) Cell mass isolated in the gastrular epithelium that will give the vestibule. (D) Cross section of a 50-day-old gastrula filled with mesenchyme. (E) Local disorganization of the gastrular epithelium at the animal pole. Nucleated cells are visible in the mesenchyme. B to D are semi-thin plastic sections (toluidine blue, pH 11.5). Abbreviations: aep, archenteric epithelium; ap, animal pole; ar, archenteron; bp, blastopore; fm, fertilization membrane; gep, gastrular epithelium; mc, migrating cells; mes, mesenchyme; N, nucleus; V, vestibule; vp, vegetal pole; vs, vitelline space. Scale bar = A and D, 250 μm; B, 50 μm; C and E, 100 μm.

but does not give any idea about the more-or-less transformed or eliminated larval phase. Furthermore, the longest sperm reported in echinoids (26 μm) was described from *Aspidodiadema jacobyi,* a species with planktotrophic development (Eckelbarger *et al.,* 1989b).

Contrary to what is considered usual for the so-called direct developers (Raff *et al.,* 1990; Wray and Raff, 1991a; Byrne, 1992), the yolky eggs of *A. cordatus* are not buoyant, in spite of their high lipid content (Magniez, 1983; Lawrence *et al.,* 1984). They sink, as do the eggs of *Patiriella* spp. (asterinid seastars with pseudo-direct development; Byrne, 1992). Lawrence *et al.* (1984) estimated the energetic density of *A. cordatus* egg to be 12.31 $J \cdot mm^{-3}$, a relatively high value, close to that of

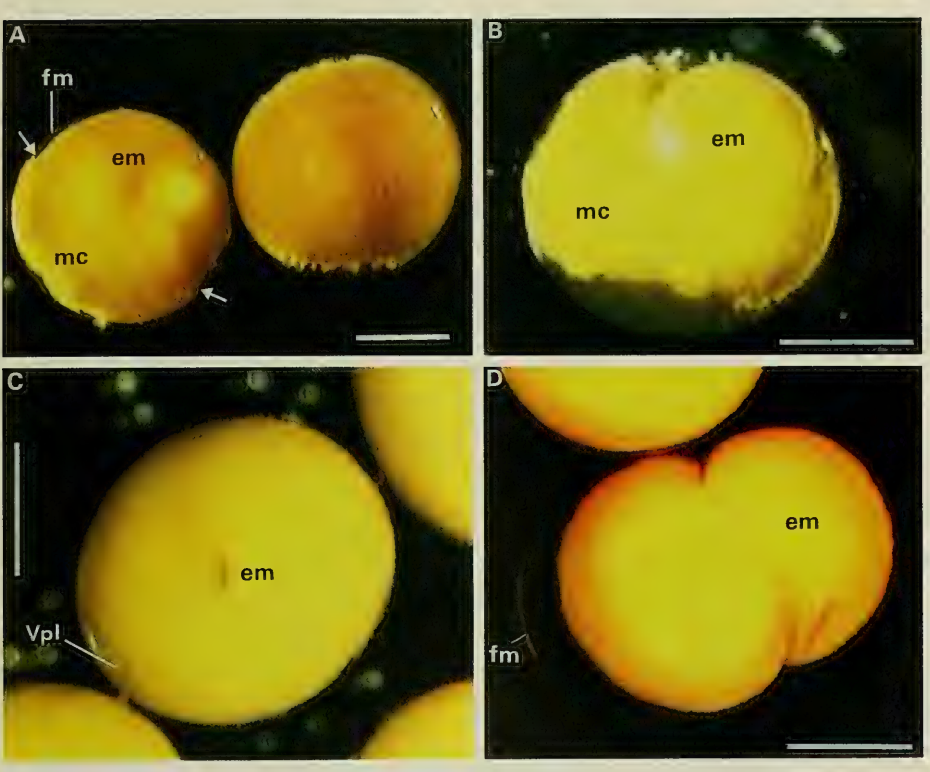

Figure 6. Gastrulation of *Abatus cordatus.* (A) Young gastrulae (32 days old) viewed from the animal pole (left) and from the side (right). The perivitelline space is filled with cells (see a nucleated cell, Fig. 6B) that migrate towards the blastopore, forming a lateral cap on half of the surface of the embryo. Arrows show the limits of the lateral cap. (B) 45-day-old gastrula surrounded by the largest number of migrating cells. Its diameter is about half the diameter of the fertilization membrane. (C) More advanced gastrula (60 days old). It takes up all the available room within the fertilization membrane. The fertilization membrane and the embryo are in contact; there is only a virtual perivitelline space, devoid of migrating cells. At the level of the blastopore, migrating cells aggregate into a vitelline plug. (D) Hatching of the gastrula, 65 days after fertilization. A to D are videotape frames. Abbreviations: em, embryo; fm, fertilization membrane; mc, migrating cells; vpl, vitelline plug. Scale bar = 500 μm.

brooding asteroid species (Lawrence *et al.,* 1984; McClary and Mladenov, 1990). Because the eggs of *A. cordatus* are spawned through dorsal gonopores, negative buoyancy assists the eggs in reaching the dorsal brood chambers.

The large, nutrient-rich egg of *A. cordatus* should provide the energy source required for its 9-month-long development. However, epidermal uptake of DOM is a possible enhancement of nutrient supply in the brood chambers of *A. cordatus,* as demonstrated for adult, larvae, and broods in different echinoderm classes (Bamford, 1982; Féral, 1985; McClary and Mladenov, 1990).

An incomplete and superficial segmentation becomes holoblastic and generates a wrinkled stereoblastula

The first horizontal constriction, which occurs 2 days after fertilization, delimits a non-nucleated vegetal structure (as revealed by Hoechst staining). This phenomenon has no equivalent among echinoderms. It is analogous to the very beginning of the formation of the polar lobe of some mollusc species in which the first cleavage is accompanied by the formation of a protusion at the vegetal pole of the egg (Verdonk and van den Biggelaar, 1983). However, experiments must be performed to determine what

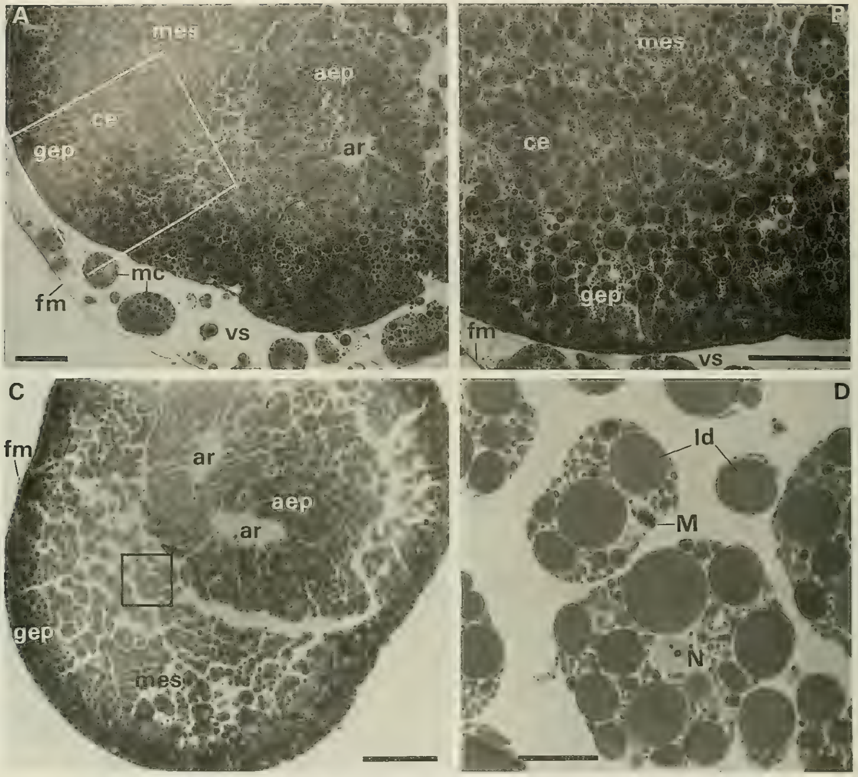

Figure 7. End of gastrulation. (A) Oblique section near the blastopore of a gastrula (between 50 and 55 days old) passing through the archenteron and one coelomic cell mass (white box). (B) Detail of the coelomic cell mass (outlined in Fig. 7A). (C) Sectioned gastrula (between 60 and 65 days old) filled with mesenchyme cells. The perivitelline space is virtually absent. The embryo takes all the room; it is in direct contact with the fertilization membrane. (D) Detail of the outlined mesenchyme cells in Fig. 7C showing interphase and mitotic (metaphase) nuclei. A to D are semi-thin plastic sections (toluidine blue, pH 11.5). Abbreviations: aep, archenteric epithelium; ar, archenteron; ce, primordial coelomic epithelium; fm, fertilization membrane; gep, gastrular epithelium; ld, lipid droplet; M, mitosis; mc, migrating cells; mes, mesenchyme; N, nucleus; vs, vitelline space. Scale bar = A and B, 100 μm; C, 250 μm; D, 25 μm.

role (other than nutrient storage) is played by this structure, which totally regresses during cleavage, in *Abatus cordatus.*

After the incomplete and superficial cleavage, the segmentation of *A. cordatus* becomes holoblastic, as in most echinoderms that produce yolky eggs (Mortensen, 1921; Raff, 1987; Olson *et al.*, 1993). This cleavage gives a wrinkled blastula, a phenomenon usually observed in lecithotrophic echinoderms (Mortensen, 1921; Fell, 1941, 1946; Okazaki and Dan, 1954; Amemiya and Tsuchiya, 1979; Byrne and Barker, 1991; Henry *et al.*, 1991; Komatsu *et al.*, 1991; Amemiya and Emlet, 1992; Byrne, 1992; McEdward, 1992; Olson *et al.*, 1993) and some planktotrophic asteroids (Henry *et al.*, 1991). In *A. cordatus,* the blastula—which is filled with mesenchyme cells—is in fact a stereoblastula. Syncytial cleavages, also resulting in stereoblastulae, were described for the holothuroid *Cucumaria glacialis* (Mortensen, 1894) and the asteroid *Fromia ghardaqana* (Mortensen, 1938) and are posited for some other echinoids (Mortensen, 1903, 1905).

Wrinkled blastulae have been described in four of the five classes of extant echinoderms (asteroids, holothuroids, ophiuroids, and echinoids) with no reference to the systematic position of the species (Fell, 1946; Patent, 1970;

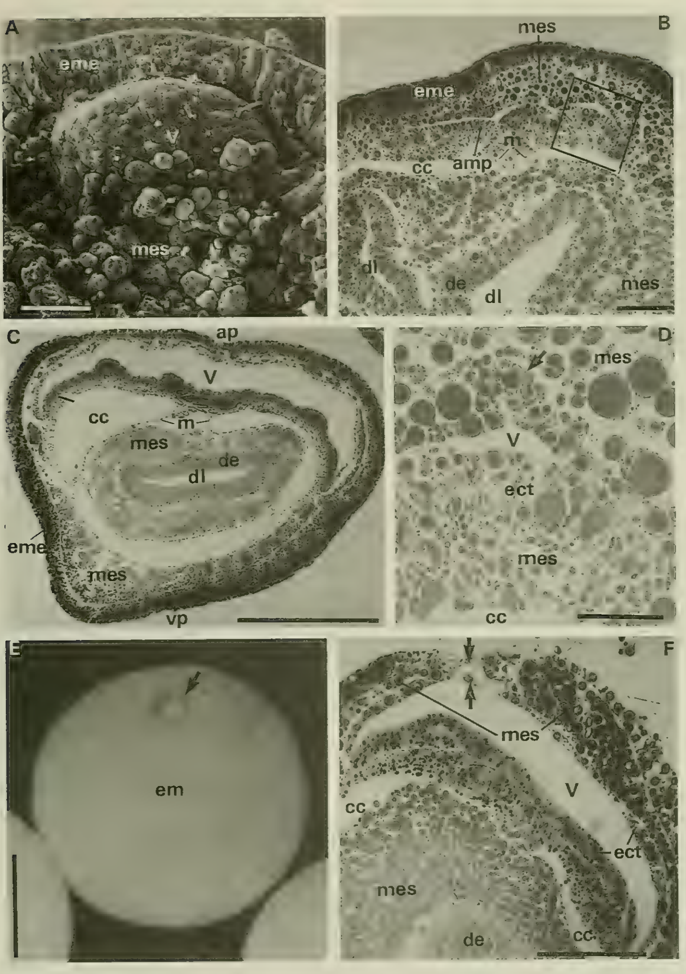

Figure 8. Post-gastrular stage of *Abatus cordatus.* (A) Developing vestibule of a newly hatched embryo (SEM). (B) Cross section through the animal part of an 80-day-old embryo. The vestibule is isolated from the embryonic epithelium and has grown in the mesenchyme. Future primary podia differentiate on the floor of the oral cavity (external fold). At this stage the future nervous system begins to be visible (internal fold). The embryonic gut is suspended in the coelomic cavity by mesenteries. (C) Cross section of a 100-day-old embryo. The vestibule is at its maximal size. (D) Detail of the black box in Fig. 8b showing an outgrowth (arrow) of the ceiling of the vestibule towards the external epithelium. (E) Late embryo (110 days old). The vestibule has opened (arrow). Details of the oral-surface-like buds of podia or spines are visible through its very thin ceiling (light micrograph). (F) Cross section through the opening (arrows) of the vestibule of a 100-day-old embryo. B to D are semi-thin plastic sections (toluidine blue, pH 11.5). F is an autoradiographic semi-thin plastic section (toluidine blue, pH 11.5; see Schatt, 1988). Abbreviations: amp, primary podia; ap, animal pole; cc, coelomic cavity; de, endoderm that will give the digestive epithelium; dl, digestive lumen; ect, ectoderm; em, embryo; eme, embryonic epithelium; m, mesentery; mes, mesenchyme; V, vestibule; vp, vegetal pole. Scale bar = A, 100 μm; B and F, 250 μm; C and E, 500 μm; D, 10 μm.

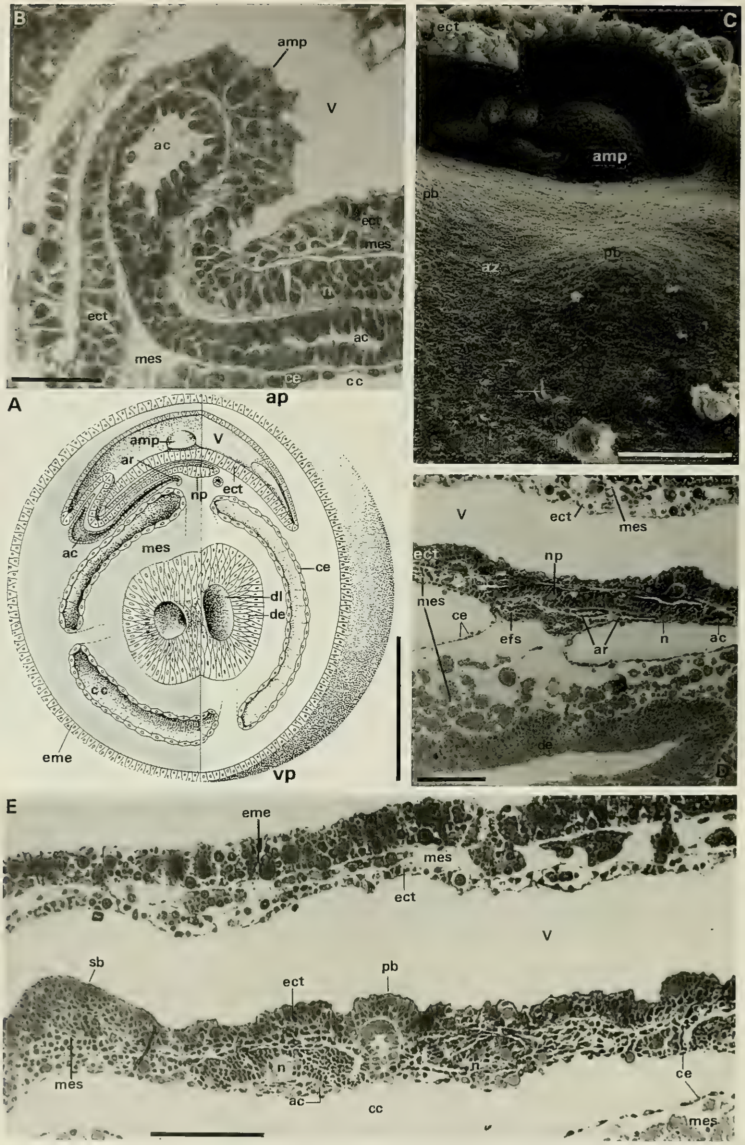
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Figure 9. Post-gastrular stage of *Abatus cordatus* (100 days after fertilization). (A) Reconstruction of the embryo passing through an ambulacral and an interambulacral section of the oral face. (B) Section through a primary podium and the ambulacral canal. (C) Oral surface of the future juvenile viewed by SEM when the ectoderm is discarded. (D) Section through the stomodeum area of the postgastrula embryo. (E) Section through the vestibule showing podia and spine buds. Radial nerves are already visible. B, D and E are semi-thin plastic sections (toluidine blue, pH 11.5). Abbreviations: ac, aquiferous canal; amp, primordial podia; ap, animal pole; ar, aquiferous ring; az, ambulacral zone; cc, coelomic cavity; ce, coelomic epithelium; de, endoderm that will give the digestive epithelium; dl, digestive lumen; ect, ectoderm; efs, ectodermic fold of the stomodeum; eme, embryonic epithelium; mes, mesenchyme; n, nerve; np, neural plate; pb, podia bud; sb, spine bud; V, vestibule; vp, vegetal pole. Scale bar = A, 500 μm; B and C, 50 μm; D and E, 100 μm.

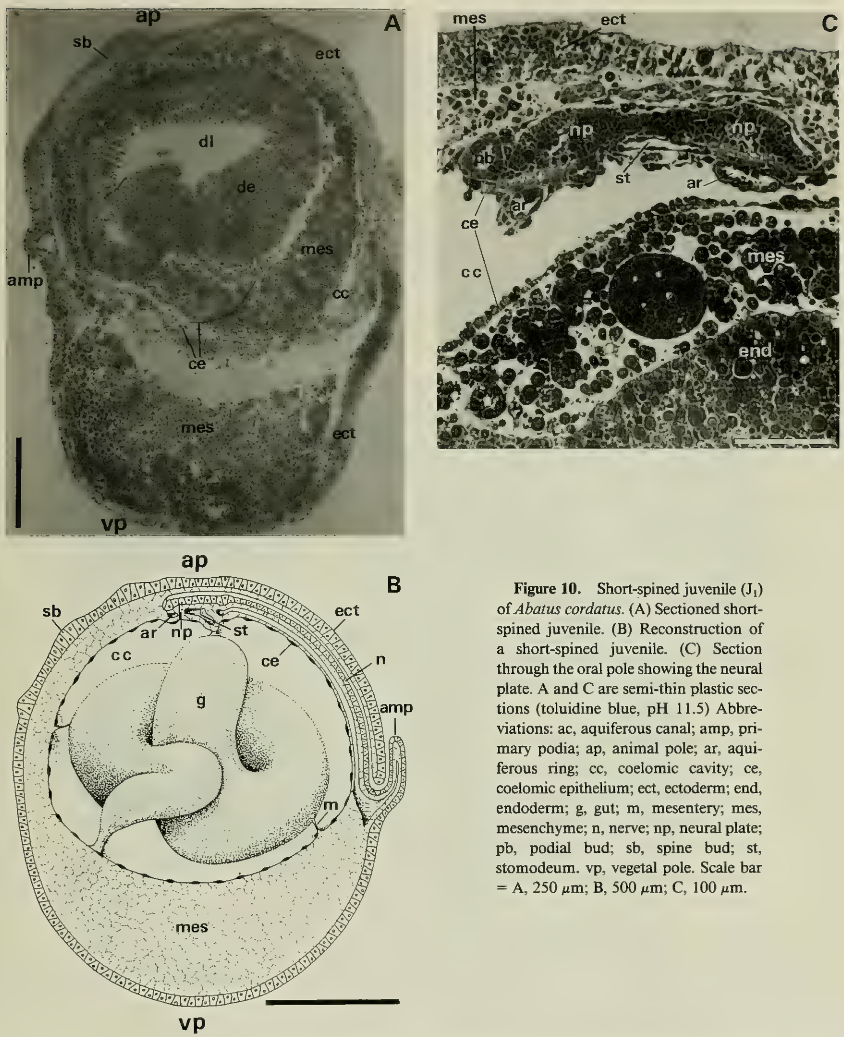

Figure 10. Short-spined juvenile (J_1) of *Abatus cordatus.* (A) Sectioned short-spined juvenile. (B) Reconstruction of a short-spined juvenile. (C) Section through the oral pole showing the neural plate. A and C are semi-thin plastic sections (toluidine blue, pH 11.5) Abbreviations: ac, aquiferous canal; amp, primary podia; ap, animal pole; ar, aquiferous ring; cc, coelomic cavity; ce, coelomic epithelium; ect, ectoderm; end, endoderm; g, gut; m, mesentery; mes, mesenchyme; n, nerve; np, neural plate; pb, podial bud; sb, spine bud; st, stomodeum. vp, vegetal pole. Scale bar = A, 250 μm; B, 500 μm; C, 100 μm.

Komatsu *et al.*, 1990, 1991; Henry *et al.*, 1991; McEdward and Janies, 1993; Cerra and Byrne, 1995). Wrinkled blastulae have been seen most frequently in starfish (Henry *et al.*, 1991). In asteroids, the smallest ovum developing through a wrinkled blastula is 125 μm in diameter (*Luidia quinaria*) and the largest is 1200 μm in diameter (*Mediaster aequalis*) as noted by Komatsu *et al.*, 1991. These authors concluded that the size of the ovum has no bearing on the occurrence of the wrinkled blastula. In echinoids, a wrinkled blastula was described for species having large ova with more or less abbreviated development (*Peronella japonica, Phyllacanthus imperialis, P. parvispinus, Asthenosoma ijimai, Heliocidaris erythrogramma;* see Komatsu *et al.*, 1991; Wray and Raff, 1991a; Raff, 1992). As emphasized by Cerra and Byrne (1995), there is no relationship between the mode of development and the presence of wrinkled blastulae. The wrinkling may be caused by the lack of space in the fertilization membrane (Chia, 1968; Byrne and Barker, 1991; Henry *et al.*, 1991; Cerra and Byrne, 1995). Our observations confirm that wrinkle formation is the result of packing an enlarged surface area in a confined space. Cell-marking experiments would be required to test whether the wrinkles result also from differences in the physical properties and

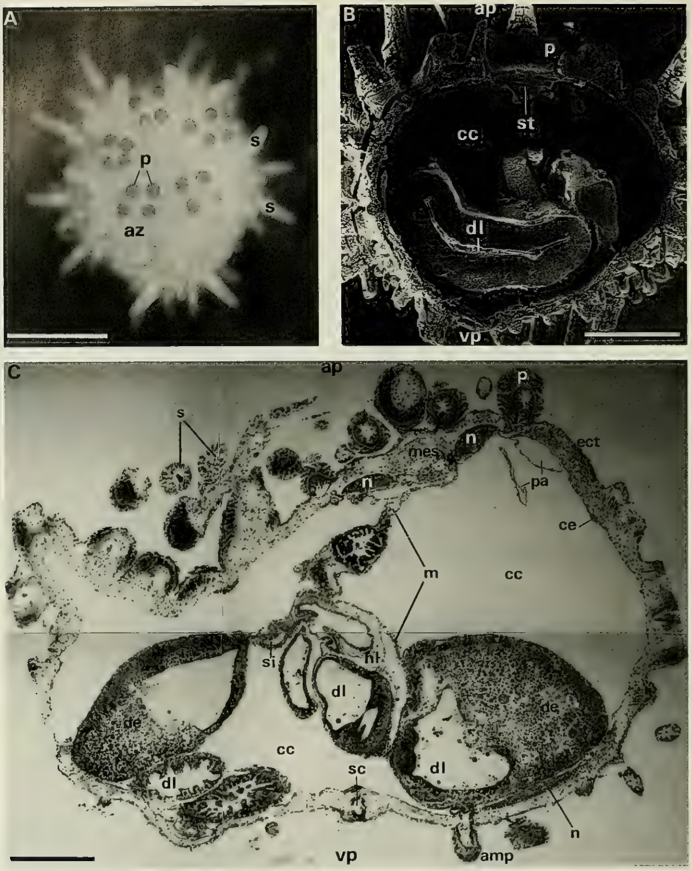

Figure 11. Long-spined juvenile (J_2) of *Abatus cordatus.* (A) Light micrograph of a juvenile (viewed from the oral pole) ready to be released from the brooding pouch. (B) SEM micrograph of a transversal opened juvenile. (C) Semi-thin plastic cross section of a juvenile (toluidine blue, pH 11.5). Abbreviations: ap, animal pole; amp, primary podia; az, ambulacral zone; cc, coelomic cavity; ect, ectoderm; de, digestive epithelium; dl, digestive lumen; hl, hemal lacuna; m, mesentery; mes, mesenchyme; n, nerve; pa podial ampullae; p, podia; s, spine; sc, stone canal; si, siphuncle; st, stomodeum; vp, vegetal pole. Scale bar = A, 1 mm; B, 500 μm; C, 250 μm.

construction of the blastular epithelium (Henry *et al.,* 1991).

In *Abatus cordatus,* wrinkles are not lost prior to gastrulation. Gastrulation may be initiated within one of the wrinkles, as observed in *Heliocidaris erythrogramma* (Williams and Anderson, 1975).

The gastrula is made of a monostratified epidermis surrounding the mesenchyme, which consists of nucleated yolky cells. The nuclei (interphase and mitotic) are quite difficult to see because the cells are big and the yolk is still very abundant. The observed lipid(?)-rich spheroids are similar in shape to the lipid droplets reported by Henry *et al.* (1991) in *Heliocidaris erythrogramma,* but they are not secreted into a cavity, which does not exist in *Abatus.* Lipid(?) droplets are inclusions of the mesenchyme cells. Epithelial cells do keep their own spheroids until the juvenile stages. No apocrine secretion is taking place at any time (see fig. 7 in Henry *et al.*).

Generally, echinoid embryos hatch from the fertilization membrane just before the beginning of gastrulation,

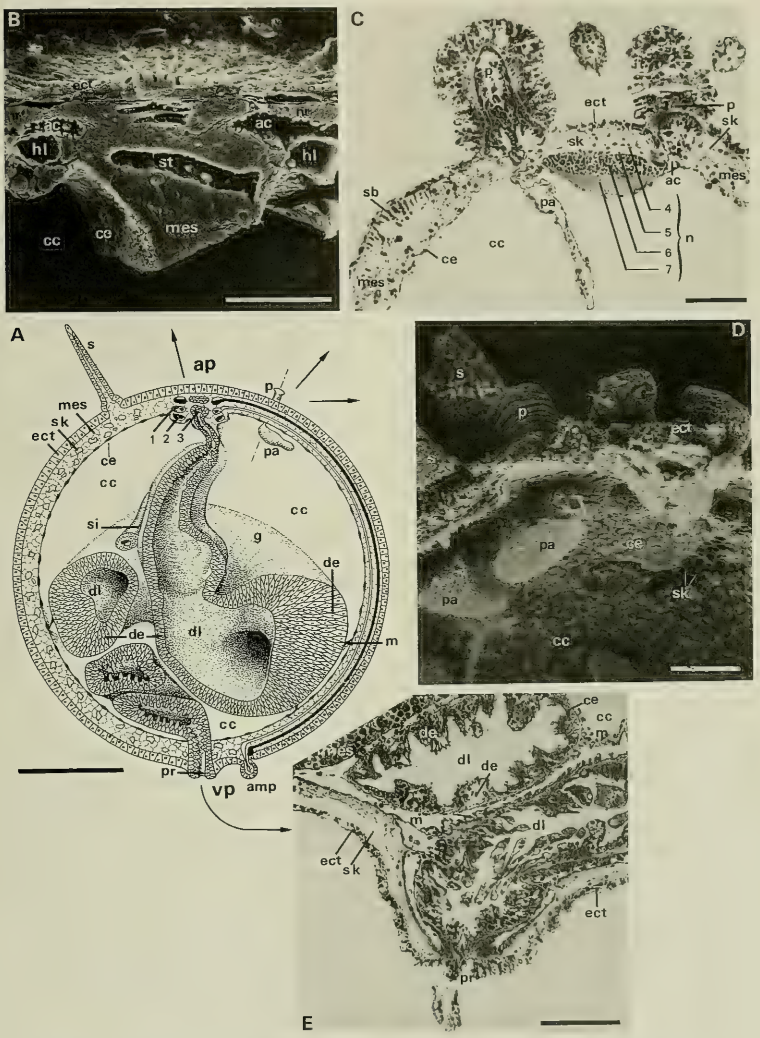
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Figure 12. Long-spined juvenile (J_2) of *Abatus cordatus.* (A) Section showing the organization of an advanced long-spined juvenile. (B) SEM micrograph of the stomodeum. (C) Transversal semi-thin plastic section (toluidine blue, pH 11.5) through an ambulacral zone. (D) SEM micrography of an ambulacral zone. (E) Semi-thin plastic section (toluidine blue, pH 11.5) through the proctodeal zone. Abbreviations: ap, animal pole; ac and 1, aquiferous canal; amp, primary podia; cc, coelomic cavity; ce, coelomic epithelium; ect, ectoderm; de, digestive epithelium; dl, digestive lumen; g, gut; h1 and 2, hemal lacuna; m, mesentery; mes, mesenchyme; n, nerve (4, epineural layer; 5, epineural sinus; 6, ectoneural layer and 7, hyponeural layer); nr, nerve ring; pa podial ampullae; p, podia; pr, proctodeum; s, spine; si, siphuncle; sk, skeleton; st and 3, stomodeum; vp, vegetal pole. Scale bar = A, 500 μm; B to E, 100 μm.

even in cidaroids with abbreviated development (Parks *et al.*, 1989; 18 h after fertilization in *Phyllacanthus parvispinus.*) In other species, hatching occurs at the beginning of gastrulation (10 h after fertilization in *Peronella lesueuri*—Mortensen, 1921; 15–16 hours after fertilization in *Heliocidaris erythrogramma*—Willliams and Anderson, 1975). In *Abatus cordatus,* hatching occurs at the end of gastrulation, *ca.* 65–70 days after fertilization. Species that hatch at the end of gastrulation or even later are found among the asteroids. In *Patiriella regularis* (indirect development), hatching by rupture of the fertilization membrane occurs 25 h after fertilization, and gastrulae become free-swimming feeding larvae (Byrne and Barker, 1991). In *Asterina gibbosa* (abbreviated development), hatching occurs 85 to 96 h after fertilization and produces a thoroughly transformed brachiolaria that rapidly adheres to the substratum (Marthy, 1980). In both cases, blastula and gastrula are hollow (Byrne and Barker, 1991; Ludwig, 1882).

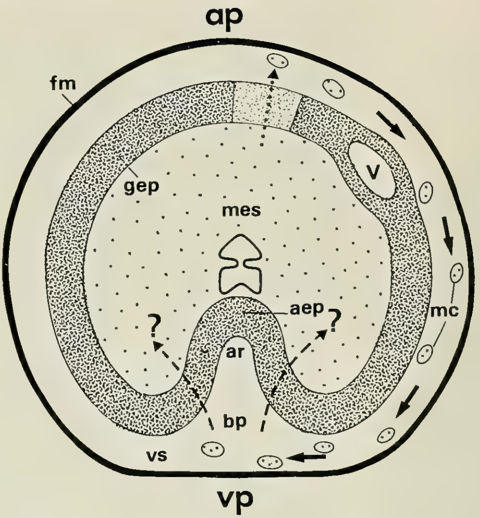

Figure 13. Hypothetical representation of perigastrulation in *Abatus cordatus.* White arrow: invagination. Dotted arrow: broadcast of mesenchyme cells through the gastrular epithelium from the mesenchyme into the vitelline space. Black arrows: migration of the mesenchyme cells in the vitelline space. Dashed arrows: hypothesized sites of ingression of migrating cells. Abbreviations: aep, archenteric epithelium; ap, animal pole; ar, archenteron; bp, blastopore; fm, fertilization membrane; gep, gastrular epithelium; mc, migrating cells; mes, mesenchyme; V, vestibule; vp, vegetal pole; vs, vitelline space.

A hypothetical new mode of gastrulation produces a pre-adult: perigastrulation

Gastrulation in echinoderms involves two simultaneous but relatively independent processes, invagination to form the archenteron and migration of the primary mesenchyme cells. As the archenteron approaches the wall of the animal region, pseudopodia-forming cells become disengaged from the archenteron and form a mass of secondary mesenchyme cells that spread over the inner surface of the adjacent ectoderm (see Burke, 1990; Burke *et al.*, 1991, for the mechanism). Modifications in gastrulation have been an important component of evolution in echinoderms and other animals (Wray and Raff, 1991b; Raff, 1992). Cell movements that occur during gastrulation establish the topological relationships between cells of the primary germ layers, setting up all the future inductions between tissues.

From the exterior, gastrulation of *Abatus cordatus* begins with smoothing of the surface. As soon as this phenomenon begins, invagination for gastrulation begins at the vegetal pole. The overall diameter does not change up to the time of hatching. Two findings suggest that this is a novel form of gastrulation involving mesenchyme cells moving out of the gastrula and reentering it during gastrulation: (1) the original diameter of the gastrula decreases while the number of migrating cells—so-called *éléments vitellins* (Schatt, 1985a; Shatt and De Vos, 1990)—increases in the perivitelline space; and (2) the diameter of the gastrula increases again at the end of gastrulation and the perivitelline space is clear before hatching. The perivitelline space is the only possible means for such movements to occur, the gastrula being continuously filled with cells (see diagrammatic Fig. 13). The fate of these migrating cells is obscure. It is possible that some reenter the embryo (ingression) at the base of the archenteron. The last ones are actually included in the vitelline plug. If two populations of mesenchyme cell (one autochthonous, the other migrating) actually exist, they may form the skeleton, the connective tissue, the muscle, or the epithelium of cavities (water vascular system, mesenteries). However, this remains speculative. Fate-mapping studies will be necessary to establish the existence and the establishment of different mesenchyme cell lineages. Experimental confirmation of perigastrulation is still needed.

Because the gastrulation of *A. cordatus* is very different from other known gastrulations, we propose to use the term *perigastrulation,* tentatively until more definitive studies are available, to describe the peculiar movement of cells within the perivitelline space during gastrulation.

Animal pole ciliary tuft and ciliated epidermis of echinoderm gastrulae (Hörstadius, 1973) have never been observed during the gastrulation of *A. cordatus,* which does not feed; these structures are also absent, or reduced, in lecithotrophic larvae (Mortensen, 1921). In contrast to the usual differentiation of the vestibule during metamorphosis (Czihak, 1971), in *A. cordatus* this process occurs at this end of gastrulation before any organogenesis. This phenomenon, unique in echinoids, allows differentiation of the juvenile to take place directly, without producing a larva.

A long organogenesis produces the miniature functional juvenile directly from the gastrula

The differentiation of the rudiment, which occurs during the post-gastrular stage of *Abatus cordatus,* is the only instance in which there is a similarity between the metamorphosis of *A. cordatus* and the metamorphosis of pluteus echinoid larva (Czihak, 1971). In *A. cordatus,* the juvenile is directly differentiated from the gastrula. The adult rudiment of *Abatus* arises from a thickening of the ectoderm and of the underlying mesenchyme that is already visible in the gastrula (65 days after fertilization) in place of the ectodermal invagination and of the left coelomic pouch in indirect developing echinoids (Hyman, 1955). In *A. cordatus,* the thickening (future vestibule) becomes isolated in the mesoderm and extends under the ectoderm of the oral pole of the embryo. The oral surface forms the neural plate, the water ring, and the five radial canals. Five primary podia arise from folds in the ectodermal floor of the cavity, just as they do in the rudiment of planktotrophic larvae (von Ubisch, 1913; Hyman, 1955; Emlet, 1988). In *A. cordatus,* the oral surface is thrust to the exterior at the beginning of the short-spined juvenile stage, when an opening develops in the ceiling of the cavity (130 days after fertilization). This process is similar to the opening of the outer wall of the vestibule in larvae of indirect developers such as *Paracentrotus* or *Psammechinus* (von Ubisch, 1913; Hyman, 1955). As a result, the morphogenesis of the rudiment of *A. cordatus* is similar to that of the other echinoids except for the formation of the cell mass that gives rise to the vestibule. Though probably independently evolved from *Abatus,* the pattern in temnopleuroids seems to be similar. The vestibule is also an ectodermally derived cell mass that is isolated from the ectoderm on the late gastrula (Fukushi, 1959, 1960). The details to substantiate this are missing from the *Abatus* series, due to the nature and timing of the material collection. In *Abatus,* the differentiation of the vestibule takes much longer than in any known case. Differences from the general case have been described in the abbreviated development of some euechinoids. In *Peronella lesueuri, Asthenosoma ijimai,* and *Heliocidaris erythrogramma,* the vestibule is more or less open to the exterior during the morphogenesis of the rudiment (Mortensen, 1921; Williams and Anderson, 1975; Amemiya and Tsuchiya, 1979; Amemyia and Emlet, 1992; Wray and Bely, 1994). In cidaroids, the absence of a vestibule appears to be typical (Emlet, 1988), and the morphogenesis of the rudiment occurs in contact with the exterior.

The characteristics of direct development in *Abatus cordatus* are not totally consistent with the hypothesis, recently advanced by several authors (Raff, 1987, 1992; Wray and Raff, 1991a; Wray and Bely, 1994), of a general acceleration, in relative time and in absolute time, in the development of adult features for the facultative feeding pluteus or the nonfeeding abbreviated pluteus. The development of *A. cordatus* is condensed (no larval morphogenesis) and presents a shift in the relative timing (heterochrony) of the appearance of the major features (archenteron, coelom + vestibule, hydrocoel), if compared to the typical echinoid development (archenteron, larval skeleton, larval gut, coelom, hydrocoel, vestibule). However, in this case the development does not accelerate in absolute time. The 250-day-long development of *Abatus* is probably a consequence of developing in cold water.

We did not observe that coeloms develop through the typical enterocoelic phenomena characteristic of most echinoderms (Czihak, 1971). The coelomic cavity of *A. cordatus* is apparently formed by schizocoely, similar to the schizocoely described in *Heliocidaris erythrogramma* by Williams and Anderson (1975). These authors described the splitting of left and right coelomic pouches immediately after gastrulation. However, the process differs from that in *A. cordatus* because the first pouches in *H. erythrogramma* develop by enterocoely. The interpretation given by Williams and Anderson was criticized by Wray and Raff (1989, 1990). Schizocoely was also hypothesized for *Gorgonocephalus caryi* (Patent, 1970) and *Amphipholis squamata* (Fell, 1946), species in which the coelomic cavity arises by splitting in the mesenchyme.

When juveniles are ready to feed (with the opening of the stomodeum 254 days after fertilization), they leave the brood pouches. This is equivalent to the settlement of the young juvenile that develops from a planktotrophic larva; settlement occurs weeks to months after fertilization (Emlet *et al.,* 1987).

The development of *A. cordatus* is strictly direct (ametamorphic). This is congruent with the fact that a histoautoradiographic study showed that there is only a unique phase of cell proliferation responsible for the first prospective organs (Schatt, 1988). In the case of the existence of larval organogenesis, before the adult organogenesis, two cell proliferations would have occurred. The adult digestive tube and the adult rudiment directly differentiate from the tissues of the gastrula. This change from a feeding larval stage to a direct development is rare. However, it occurs in ophiuroids (Fell, 1945, 1948) and asteroids (McEdward, 1992; McEdward and Janies, 1993). In order to avoid any misunderstanding, we propose to reserve the term *direct development* for species that exhibit completely direct development and the term *abbreviated development* for species that have more or less entirely eliminated the pluteus stage. The term *mesogen* was proposed for directly developing asteroids to refer to the developmental stages that occur between the end of the gastrula stage and the beginning of the juvenile stage

(McEdward and Janies, 1993). However, we kept the term *post-gastrular stage* in *A. cordatus* because it seems to us more easily understandable and more general.

To feed or not to feed?

As stated by Strathmann (1978) and Raff (1987), loss of the feeding-larval stage is irreversible and strongly correlated with egg size. Raff (1987) hypothesized that constraints existing in the development of larval stages are eliminated by abandoning the need for a feeding stage. Increasing the amount of yolk available to the embryo seems to make this possible. The archenteron of *Abatus cordatus,* first isolated within the mesenchyme, elongates and differentiates into the adult gut starting with the beginning of juvenile stage 1. This gut becomes functional shortly before the stage 2 juveniles are released from the brood chambers, about 250 days after fertilization (Schatt, 1985a). Schatt (1988) showed that the dry weight is constant (*ca.* 0.7 mg) from spawning to 165 days after fertilization (half of the juvenile stage 1). Then, it increases to reach *ca.* 2.5 mg for the juvenile stage 2, at the moment where they are released before the opening of the mouth. The increase of weight of *A. cordatus* is essentially due to the differentiation of the adult skeleton (Schatt, 1988) and is congruent with our microscopic observations. These findings may indicate that yolk provides enough nutrients to maintain the embryos and juveniles (Lawrence *et al.,* 1984) or that very little metabolic activity is occuring for most of the development. However, McClary and Mladenov (1900) reported evidence for nutrient translocation to brooded juveniles of the sea star *Pteraster militaris* as well as evidence of DOM uptake. This needs to be demonstrated experimentally for *A. cordatus* (see Fenaux, 1982, for a review).

Do other completely direct developing echinoids exist?

Emlet (1990) counted 14 lineages of echinoids with nonfeeding development. Of these, eight involve brood-protection (Cidaroida, Temnopleuroida, Echinoida, Spatangoida [including Schizasteridae and Brissidae], Holasteroida, Cassiduloida, Clypeasteroida). This represents 7% (15 spp.) of the regular echinoids and 35% (24 spp) of the irregular echinoids. Only a few of them are completely direct developers.

It is easy to predict that other subantarctic and Antarctic schizasterids will be found to be true direct developers. Different stages have already been described, particularly the two short- and long-spined juvenile stages (*Abatus* spp, *Amphipneustes* spp, *Parapneustes abatoides,* Agassiz, 1874; Verrill, 1876; Mortensen, 1910, 1951; Bernasconi, 1969; Larrain, 1973; Magniez, 1980; Schatt, 1985a; Pearse and McClintock, 1990; Schinner and McClintock, 1993; see also Schatt and Féral, 1991, for brooding schizasterid references and Poulin and Féral, in press, for other Antarctic echinoids). One can also add the abyssal Antarctic species *Delopatagus brucei,* formerly classified as a member of the Asterostomatidae but now considered to be in the Schizasteridae, for which De Ridder *et al.* (1993) mentioned the presence of juveniles in dorsal brood pouches. Féral *et al.* (1994) showed by means of the phylogenetic analysis of partial sequences of 28S rRNA that *Delopatagus* fell into the brooding schizasterid group, clustering with *Amphipneustes* spp., a genus closely related to *Abatus.*

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In situ Rates of Fertilization Among Broadcast Spawning Gorgonian Corals

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Abstract. Fertilization rates among marine benthic taxa have implicitly been assumed to be uniformly high in most analyses of life history evolution, but *in situ* fertilization rates during natural spawning events are rarely measured. Fertilization rates of the Caribbean gorgonians *Plexaura kuna* and *Pseudoplexaura porosa* were measured at a site in the San Blas Islands, Panama, by collecting eggs downstream of colonies during synchronous spawning events during the summer months in the years 1988–1994. Eggs collected by divers were incubated, and the proportion of eggs that developed was determined. Proportions of eggs developing suggest fertilization rates that vary from 0% to 100%. Monthly means ranged from 0% to 60.4%. Failure of gametes to develop can be attributed to sperm limitation, as eggs collected during spawning had higher fertilization rates if incubated with an excess of sperm. *Plexaura kuna* fertilization rates were highest during the July spawning events. Fertilization of *Plexaura kuna* eggs was usually lower during the first two nights of the 4–6 night spawning event. The proportion of eggs being fertilized when collected from a given place and time was highly variable, with one peak in the frequency distribution at or below 20% fertilization, and a second group of samples with greater fertilization rates. High variance in fertilization rates is evident at all levels of analysis: between replicate samples, times within nights, and among nights and months. This variance can be attributed to a combination of the effects of heterogeneity in the water column as gametes are diluted, spawning behavior of the gorgonians, and the current regime. Fertilization rates are often low and may represent a limiting step in recruitment during some years. Low fertilization rates may also be an important component of the life history evolution of these species.

Introduction

One of the primary goals of benthic ecology has been the determination of the factors limiting populations. For most of this century, such efforts have been concentrated on post-recruitment events in the life history of the organism (*i.e.*, Connell, 1961, and hundreds of subsequent studies). Missing from such analyses are the processes that directly or indirectly affect the number of larvae that settle. The importance of such processes was recognized by many authors (*e.g.*, Thorson, 1946), but the difficulties of measuring phenomena such as survival of larvae in the water column led to research that virtually ignored larval ecology in favor of the more knowable post-settlement dynamics of populations. More recently, larval ecology has been rediscovered (Young, 1990; Grosberg and Levitan, 1992), and a wide variety of work has documented the importance of larval dynamics and the circumstances under which recruitment may limit populations (Connell, 1985; Gaines and Roughgarden, 1985; Gaines *et al.*, 1985; Roughgarden *et al.*, 1988).

As in the earlier treatments, most studies of larval ecology have also glossed over a potentially limiting but seemingly unknowable component of the life cycle of broadcast-spawning taxa: the proportion of gametes that are fertilized and that complete early development. This bias reflects the general difficulties of determining the fate of gametes and embryos after they are released into the water column.

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Thus, most analyses of benthic invertebrate reproductive strategies have focused on fecundity and the subsequent survival of the larvae as the rate-limiting steps controlling successful recruitment (for instance, Vance, 1973; Strathmann, 1978, 1985; Strathmann and Strathmann, 1982; Roughgarden, 1989). Assumptions that are implicit in these analyses are that eggs are readily fertilized, that fertilization rates are relatively uniform over time and space, and that the proportion of eggs fertilized is independent of reproductive strategy.

The assumption that fertilization is uniformly great may be incorrect for many species, and a growing body of literature suggests that fertilization has the potential to be a limiting step in the life histories of some benthic species. Pennington (1985), Yund (1990), Levitan (1991), Levitan *et al.* (1991, 1992), Babcock *et al.* (1992, 1994), Brazeau and Lasker (1992), Oliver and Babcock (1992), Benzie *et al.* (1994), Benzie and Dixon (1994), Yund and McCartney (1994), and Levitan (1995) have experimentally demonstrated the potential for sperm limitation in the fertilization of a variety of benthic species. These measurements, in concert with the modeling efforts of Denny (1988) and Denny and Shibata (1989), suggest that eggs are often sperm limited.

Measurements of fertilization rates from naturally spawning events have yielded rates that are substantially below 100% (Table I). The most extreme of those rates have been those of *Briareum asbestinum,* which has been observed over multiple years and sites. Observed rates were as low as those suggested by experimental data (Brazeau and Lasker, 1992). However, in many cases fertilization rates during natural spawning events have been surprisingly great, as among *Acanthaster planci* (Babcock and Mundy, 1992). Although the rates presented in Table I are generally below 100%, most are also well above 0%. Apparent fertilization rates that are lower than 100% could be a function of egg viability or biases introduced by sampling techniques. The fertilization rates that have been reported, although consistent with the hypothesis of sperm limitation, do not demonstrate sperm limitation. Furthermore they suggest that sperm limitation may be taxon-, site-, and time-dependent. If these restrictions held, sperm limitation could contribute to the differential success of populations and species at differing sites.

Most field measurements of fertilization rates have also been restricted to either single spawning events or sites. Thus both the extent and source of variation in fertilization rates are unknown for most taxa. Characterization of this variation, when it has been conducted, has proven to be extremely interesting. Brazeau and Lasker (1992) examined fertilization rates of *Briareum asbestinum* at two sites in Panama over two years. They reported 0.0% fertilization rates from one of those sites (*i.e.,* complete reproductive failure), suggesting that some sites with seemingly healthy populations may have little impact on the survival of a species. Petersen and colleagues (Petersen, 1991; Petersen *et al.,* 1992) reported on fertilization rates of wrasses, both across reefs and across days, and they identified environmental conditions that may enhance fertilization success and explain variance in fertilization success. These results suggest that fertilization success, by controlling the relative contribution of individuals, may be an important variable in the evolution of the life history of a species.

Table I

Fertilization rates determined from in situ *collections of spawned eggs or from incubations of eggs with sea water samples*

Species	Fertilization rate	Reference
Briareum asbestinum (gorgonian)	<0.01–6.5%	Brazeau and Lasker, 1992
Montipora digitata (coral)	0.2–49.0%	Oliver and Babcock, 1992
Acanthaster planci (asteroid)	23–83%	Babcock and Mundy, 1992
Actinopyga lecanora (holothurian)	67–78%	Babcock *et al.*, 1992
Bohadshia argus (holothurian)	0–96%	Babcock *et al.*, 1992
Cucumaria miniata (holothurian)	92 (1–100%)	Sewell and Levitan, 1992
Holuthuria coluber (holothurian)	9–83%	Babcock *et al.*, 1992
Halichoeres bivittatus (wrasse)	20–100%	Petersen, 1991
Thalassoma bifaciatum (wrasse)	76% (0–100%)	Petersen *et al.*, 1992

In this paper we report fertilization rates for the broadcast spawning Caribbean gorgonians *Plexaura kuna* and *Pseudoplexaura porosa* at a site in the San Blas Islands, Panama. We document variation in fertilization rates over spawning events that occurred over 15 months during the period 1988–1994, and we show that the low fertilization rates can be attributed directly to sperm limitation at this site.

Materials and Methods

Fertilization success of the gorgonians *Plexaura kuna* (previously discussed as *Plexaura* A, Lasker *et al.,* 1996) and *Pseudoplexaura porosa* was measured by determining the proportion of spawned eggs which initiated development. *P. kuna* is a gonochoric, broadcast spawner that releases its eggs during 4–6 day spawning events that occur four days after the full moon during the months of June–September or May–August depending on the timing of the full moon (Brazeau and Lasker, 1989). In Panama spawning is restricted to a 60-min period starting 0–40 min after sunset, and most of the spawning occurs during the middle 30 min of the release period. *Pseudoplexaura porosa* is also a gonochoric broadcast spawner, and it spawns over a 3-day period. Its spawning event usually overlaps with *P. kuna* on 2–3 nights. A second, distinct *Pseudoplexaura* sp. was observed on two nights and collections of its eggs were also made when colonies were observed spawning. Collections of all species were made at the northeast corner of Korbiski Reef, a small reef located near the San Blas field station of the Smithsonian Tropical Research Institute (STRI). Gorgonians, and *P. kuna* in particular, dominate the benthos in this area (see descriptions in Lasker and Coffroth, 1985; Lasker, 1990). Collections were made at arbitrarily selected locations on the reef at depths of 2–5 m.

Gametes used in experiments were collected from colonies maintained in 18-l aquaria at the STRI field station. Colonies were collected several days prior to spawning and transferred to aquaria in which the seawater was replaced 5–7 times daily. Colonies maintained in this fashion spawned on the same nights as *in situ* colonies, but spawning in aquaria usually lagged behind *in situ* spawning by 30–90 min.

We assayed released eggs for fertilization during spawning events between 1988 and 1994. Collections during 1988–1991 were made at arbitrarily selected positions immediately downstream of female colonies that were releasing eggs. Divers were usually 1–3 m from the colony and collected the large (>600 μm diameter) eggs in 60-ml syringes. Starting in 1992, eggs were collected by SCUBA divers positioned along transects several meters downstream of large clusters of colonies releasing eggs. In all years sites for collection were chosen to minimize collection of eggs from other colonies and other species. Divers surveyed the collection areas for eggs from other colonies both before and after the collections. Collections believed to be contaminated with eggs of unknown origin were not considered in the analyses. Upon collection, eggs were either transferred to a 1-liter-capacity plastic bag (1988–1991) or were left in the syringes (1992–1994). After 0.5-2 h, eggs were counted, placed in seawater that had been collected prior to the start of spawning (henceforth referred to as sperm-free seawater) and then incubated in 120-, 500-, or 1000-ml containers. Concentrations of eggs were kept below 500 in 1000-ml containers, 200 in 500-ml containers, and 75 in 120-ml containers. All containers were soaked in seawater for at least 2 weeks prior to use, and then rinsed and dried prior to use. The containers were suspended in mesh bags off of a dock at the STRI field station. The containers, which were slightly buoyant, bounced with the mild sea swell at the laboratory site, and the eggs, which were buoyant, were distributed throughout the water in the container. Seawater in the containers was changed at 12 h and when applicable at 36 h. The number of developing embryos observed either 12 h (1993, 1994) or 36 h after spawning (1988–1992) was used as our estimator of the number of eggs that had been fertilized. Number of embryos at 12 h was determined using a dissecting scope at 20×; number of embryos and/or planulae at 36 h was determined without magnification.

In 1993 and 1994 we also collected water samples simultaneous with our egg collections in order to measure the "fertilization potential" of the water. These samples were transported back to the STRI field station where they were used to incubate eggs that had been collected out of aquaria that contained female colonies alone. Incubations were started 0.5–2 h following collection of the water samples. *Plexaura kuna* sperm remain viable for 2–4 h (Lasker, unpub.). As in the field samples, eggs were assayed the following morning to determine the proportion fertilized.

In order to determine whether *in situ* fertilization was sperm limited, a series of paired samples were collected in August 1992. Eggs were collected on the reef in syringes as described above. Eggs from two syringes were pooled and counted and one half of the eggs were then incubated in sperm-free water, while a second half of the eggs were incubated using water from an aquarium containing a male colony. After 12 h the number of developing embryos was determined in each sample. Sperm density was not determined, but in more recent experiments we have found that sperm densities in such tanks are typically $>10^5$ sperm/ml.

Our collecting techniques incorporated several potential biases, and a series of experiments were conducted to assess biases in our estimates of fertilization. Eggs that are collected in syringes are maintained in water that presumably contains sperm. Fertilization is a function of sperm density for *Plexaura kuna* (Lasker and Stewart, 1993). Sperm density in the water column would presumably steadily decline as a function of turbulent mixing. Thus eggs would normally be exposed to ever decreasing concentrations of sperm as they were transported off the reef. Eggs collected and then maintained in syringes for 0.5–2.0 h could have elevated fertilization rates. As a test of such an effect, paired samples of eggs were collected by divers stationed next to each other. These samples were immediately carried to an overhead boat. In an initial experiment, in July 1993, one of the two samples was immediately diluted 20 times and then both samples allowed to develop overnight. In August 1993 we further tested for a "syringe effect" by taking one of the paired samples and immediately washing it with sperm-free water. The eggs were rinsed with 200 ml of seawater in a 150-μm-mesh Nitex filter device that kept the eggs submerged and off the screening at all times. The eggs were then transferred to a 120-ml container and thereafter treated like all other field-collected eggs.

An additional set of washing experiments was also conducted using virgin eggs collected from aquaria that contained only female colonies. Virgin eggs were (1) incubated with sperm overnight without any washing [control]; (2) incubated for 1 min in seawater containing sperm, then washed and incubated with sperm-free seawater [washed]; (3) incubated for 1 min in sperm-free seawater, then washed and incubated overnight in water containing sperm [control for effects of washing on eggs]; and (4) incubated with sperm for 1 min, then washed and incubated overnight in seawater containing sperm [control for effects of washing on fertilized eggs]. These treatments assessed whether the rinsing procedure damaged eggs and reduced apparent fertilization rates.

To assure the identity of collected eggs, samples were gathered immediately downstream of spawning colonies. Thus eggs were collected within 1–5 m of their source colony and, depending on current speed, were probably being collected within 0.5–3 min of release. We have not observed any delay in maturation of eggs released by *Plexaura kuna* colonies (unpub. data). However, in other species, fertilization rates are affected by the amount of time exposed to sperm (Levitan *et al.,* 1992). Therefore our observed fertilization rates could be artificially low. To test for such an effect we conducted two surveys. On August 10, 1993, two divers collected eggs being released by a single *Pseudoplexaura* sp. colony. This colony was the only female colony releasing eggs at the time of the collection (1955–2058) and identity of the eggs is virtually certain. One diver collected eggs from approximately 1 m away and the second from 6–8 m downstream. Eggs were collected and incubated as previously described. Numbers of developing embryos were determined the following morning.

A second assay for potential downstream fertilization was made by collecting seawater downstream of the reef and then using that water to incubate virgin eggs. Downstream water was obtained by releasing a drogue buoy during the period of peak egg release and then collecting water from around the buoy 5–20 min later. The water was transported back to the field station and replicate samples of 50 virgin eggs incubated in the water in a 120-ml container. The drogue could only be deployed on nights when the prevailing currents would not wash the buoy onto the reef. On all nights a water sample was collected from the reef and used to incubate virgin eggs.

The proportion of eggs that had developed was assayed at 12 h (1993–1994) and 36 h (1988–1992) after spawning. This estimator of fertilization could underestimate fertilization success by incorporating post-fertilization mortality. In 1991, 20 groups of 50 eggs were incubated in water containing sperm and the number observed developing was determined at both 12 and 36 h after the start of the incubation.

Results

Fertilized eggs began cleaving several hours after exposure to sperm. After 4 h embryos were at the 16–64 cell stage of development. After 12 h individual cells were no longer visible and the stereoblastula exhibited a characteristic raisin-like wrinkled appearance. Between 12 and 36 h, embryos elongated and took on the appearance of a planula, but only a few of the planulae were actively swimming at 36 h. Twenty groups of 50 eggs that were incubated with sperm had mean survivorship of 45.6% (S.D. = 2.2) at 12 h and 37.0% (S.D. = 5.9) after 36 h. The decrease in survivorship was significant ($F = 46.5$, df = 1, 19, $P << 0.001$). The fertilization rates observed at 12 h ranged only from 44 to 50%, and over this range there was not a significant relationship between the initial fertilization rate and the decline over the subsequent 24 h (fertilization at 12 h *vs* 36 h, $r^2 = 0.11$). However, the result does indicate that mortality of developing eggs during the 1988–1992 observations led to an underestimate of fertilization of 9%.

Simple 20-fold dilution of the water in which the eggs were collected had no effect on fertilization (Fig. 1), indicating either that the dilution was insufficient to elicit a response or that there were only minimal numbers of post-collection fertilizations. Diluted and undiluted incubations were split evenly between Diver 1 and Diver 2 collections. Variance between samples, including simultaneous and adjacently collected paired samples, was extremely high. Between-sample variance made up 66% of the total variance among those samples, with between-pair (*i.e.,* time) variance contributing 34% of the total variance. The washing experiments indicated that holding eggs in syringes with seawater from the reef had a small but significant effect on fertilization rate (Fig. 2). Measuring the effect of washing was made difficult by the high level of sample to sample variance (std. dev. of fertilization rates among control samples, 20%; percent of variance explained by error term, 34%; and percent of variance explained by between-paired samples, 65%). On average, washing reduced fertilization rate by 4%, but as is evident from Figure 2, the variance in the effect was large [std. dev. of (control fertilization rate − washed fertilization rate) = 17%]. When analyzed as a two-way ANOVA (arctan transformed values) there was no significant difference between washed and control fertilization rates, and the slope determined by RMA regression analysis (Niklas, 1994) did not differ from 1.0 (Fig. 2). (The experiments for filtration effects indicate that eggs could still be fertilized following filtration. Eggs in the control had a fertilization rate of 67%; eggs that were added to sperm-con-

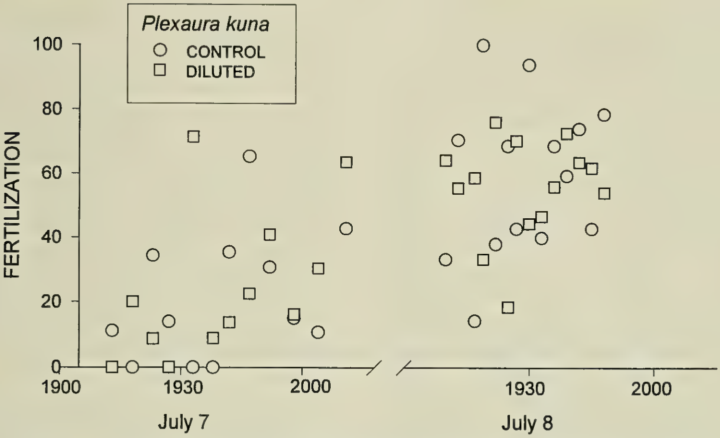

Figure 1. Proportions of *Plexaura kuna* eggs fertilized in individual samples collected on two nights at Korbiski Reef in 1993. Diluted and undiluted samples were collected from within 1.5 m of each other at identical times. Half of the samples were diluted 20-fold after collection, but dilution had no effect on fertilization rates (see text).

taining seawater but immediately washed had a fertilization rate of 15%, indicating that washing could prevent post-collection fertilizations. Eggs that were washed and then incubated with sperm-containing water had a fertilization of 60% and those that were exposed to sperm, washed and then again incubated with sperm had a fertilization rate of 71%. These latter controls indicate that mechanical effects of washing on both fertilized and unfertilized eggs were negligible.)

Fertilization rates in the water column as eggs drifted away from the reef had minimal effect on our field estimates of fertilization. *Pseudoplexaura* sp. eggs that were allowed to float in the water column for an additional 5–7 m after release did not have greater fertilization rates than eggs collected 1 m downstream of the colony (38% at 1 m *vs.* 30% at 6–8 m; *t*-test $P > 0.20$, $n = .013$ paired samples). Water collected immediately downstream of the reef during *Plexaura kuna* spawning events was often capable of fertilizing eggs, but fertilization rates in those experiments never exceeded 4%. Those values are probably maximal estimates of downstream fertilizations, because the water samples were taken only on days with low current flow (approx. 2.5 cm/s), when mixing of the sperm would be lowest, and because incubations with those water samples (*i.e.*, exposure to sperm) lasted 12 h instead of the minutes of exposure that would occur naturally.

The number of samples obtained during each of the days/months/years was highly variable (Table II). Differences between years and months reflect differing collecting effort and differences between days within months reflect the availability of eggs at the Korbiski site. The number of days sampled (3–4) in each month usually reflects our inability to collect unambiguously mono-specific *Plexaura kuna* collections on the last two days of the spawning event when *Pseudoplexaura porosa* also spawned.

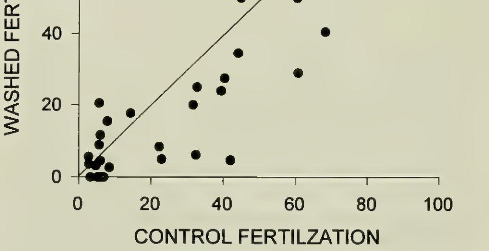

Figure 2. Effects on *Plexaura kuna* fertilization rates of washing eggs immediately following collection.

Table II

Collection sites and numbers of samples collected at Korbiski Reef, Panama, during spawning events

Date	Site	Number of days	Number of samples	Number of eggs
June 1988	A	2	2	108
July 1988	A	2	8	1,084
August 1988	A	1	2	48
June 1989	A	3	10	678
July 1989	A	6	26	3,526
June 1990	A	2	4	318
July 1990	A	3	13	2,881
August 1990	A	5	13	3,192
May/June 1991	A	3	16	247
June/July 1991	A	5	29	3,132
August 1991	A	1	2	209
July 1992	A	4	145	3,251
August 1992	A	3	36	1,983
July 1993	T	4	109	2,802
August 1993	T	4	84	3,049
June 1994	T	4	60	2,119
July 1994	T	5	61	3,401
August 1994	T	3	62	2,230

A, arbitrarily selected colonies; T, transect located downstream of colonies.

Fertilization rates of *Plexaura kuna* eggs from individual samples were most commonly below 50% and were extraordinarily variable. Figure 1 presents fertilization rates of eggs collected on two days. Fertilization rates on those days were among the highest observed in the study. Fertilization rates tended to rise at the start of spawning, but as is evident in Figure 1 this pattern is itself variable. The increase suggests that either sperm concentration or egg receptivity changed over the course of the spawning event. The data from the 1- and 6-m *Pseudoplexaura* collections suggest no short-term change in egg receptivity, and in laboratory experiments we have been able to fertilize *P. kuna* eggs shortly after they were released (unpub. data). These data suggest that the rise in fertilization rates observed early in the spawning event was probably driven by the release and accumulation of sperm in the water column.

As is evident in Figure 1, regardless of whether mean fertilization rates were great or small, samples taken by individuals spaced only meters apart could vary by 100% or more. The samples illustrate the extraordinary variability in fertilization rates between samples that were taken within 0.5–2 m of each other. Similarly, collections taken by the same individual at the same location varied between collections as well. (An average collection required 2–5 min to gather.) When averaged across days, much of the variability disappeared and there was a general tendency for the first and last day(s) of spawning in each month to have lower fertilization rates than the middle 2–3 days (Fig. 3).

Monthly average fertilization rates of *Plexaura kuna* eggs were well below 100% and were also highly variable. In some months there was virtual reproductive failure of the Korbiski Reef *P. kuna* population (Fig. 4). Fertilization rates were highly variable over both months and between years. The highest fertilization rates occurred during July 1993 spawning events. The values in 1993 and 1994 tend to be among the highest, which may be attributed to the shorter incubation and greater embryo survivorship at 12 h compared to 36 h. However, low fertilization rates were observed among those two years as well. The lowest fertilization rates occurred in August 1988, and June, July, and August 1990 (0, 4.1, 3.1, and 2.9%, respectively).

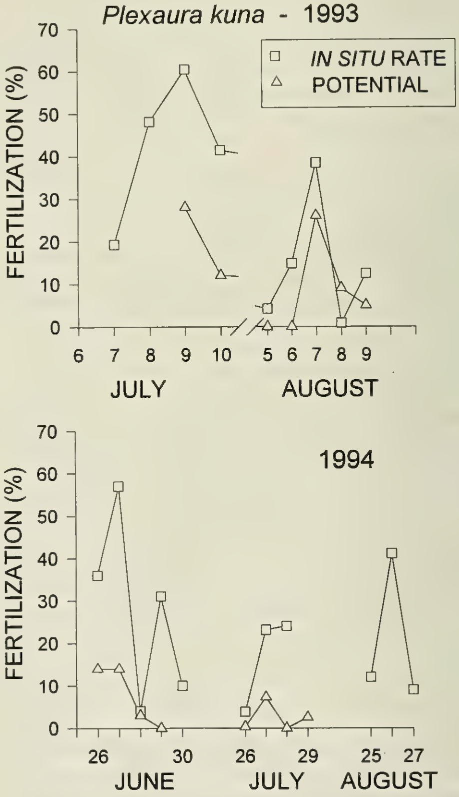

Figure 3. Daily averages of proportion of eggs fertilized in samples of eggs that were collected on Korbiski Reef (*in situ*) or were incubated with water from Korbiski that was collected at the same time as the *in situ* egg samples (potential).

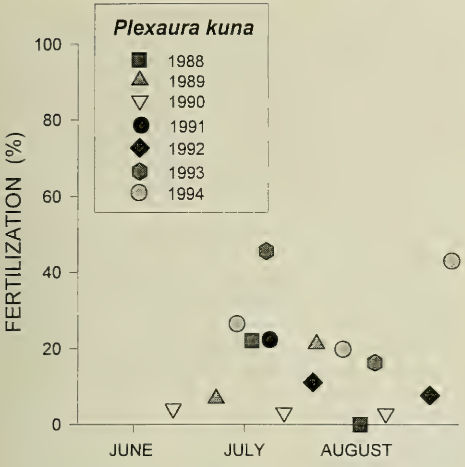

Figure 4. Proportions of *Plexaura kuna* eggs of collected eggs that were fertilized during spawning events at Korbiski Reef between 1988 and 1994. Values are weighted averages of data from 1–5 days of collections during each month.

Variance in fertilization rates was driven by the tendency for eggs from individual samples (*i.e.*, single syringes) to have somewhat bimodally distributed fertilization rates (Fig. 5). The frequency distribution of proportion of samples having a given level of fertilization is usually characterized by a large peak at or below 20% fertilization and either a very broad tail at higher fertilization rates or in a number of cases a second, smaller peak at a greater fertilization rate. This pattern was much less pronounced in 1993 when relatively high rates of fertilization were observed. However the pattern returned when samples were partitioned between months or days.

Measures of fertilization potential were significantly correlated with the daily average *in situ* rates (Fig. 3, $r = 0.64$). Fertilization potentials were almost always lower than the field rates, which may reflect a decrease in sperm activity associated with the 0.5–1 h delay between collection and the introduction of eggs to the water sample. There was no significant correlation when the fertilization potential of individual samples was compared to the fertilization rate of simultaneously collected eggs (Fig. 6).

Eggs collected *in situ* and then incubated with additional sperm always had greater fertilization rates than controls that were not supplemented with additional sperm (Fig. 7). The increase was dramatic as among controls the greatest fertilization rate was 38% and the lowest fertilization rate among the treated samples was 68%. The average difference between paired control and treatment was 72%.

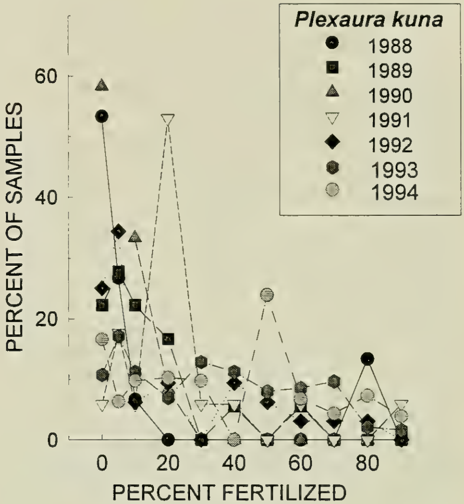

Figure 5. Frequency distribution of fertilization rates of *Plexaura kuna* eggs collected in samples from Korbiski Reef in the San Blas Is., Panama.

Pseudoplexaura porosa fertilization rates were determined on 19 days between 1990 and 1994. Mean fertilization rates over those days were 32% (1990), 85% (1992), 39% (1993), and 50% (1994). Fertilization rates were bimodally distributed as among *Plexaura kuna* samples but, for *Pseudoplexaura porosa* samples, the second peak occurred at a greater fertilization rate and included a greater

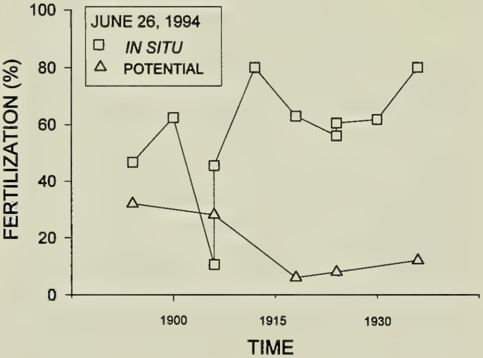

Figure 6. Proportions of eggs fertilized in individual samples of eggs that were collected on Korbiski Reef (*in situ*) or were incubated with water from Korbiski that was collected at the same time as the *in situ* egg samples (potential).

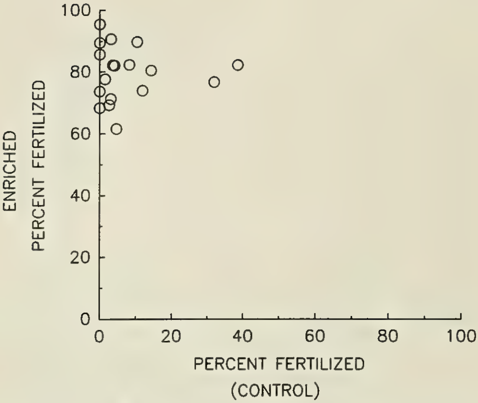

Figure 7. Fertilization rates among *in situ* samples of eggs incubated with and without an excess of sperm. Dotted line represents expected relationship if enrichment with sperm had no effect on fertilization rate.

percentage of the samples. Fertilization rates between days exhibited the same degree of variance as shown among the *P. kuna* samples (Fig. 8).

Discussion

Regardless of whether one looks at the data from individual samples or from daily or monthly averages, both *Plexaura kuna* and *Pseudoplexaura porosa* eggs have fertilization rates that were almost always well below 100% and often approached 0%. Indeed, the majority of *P. kuna* samples collected at Korbiski had rates <20%. On virtually every night, there were samples with near-zero fertilization rates, and on some occasions rates were uniformly low, as in 1990. During 1988–1991, samples were collected immediately downstream of arbitrarily selected colonies, and some of the low rates may simply be a function of the colonies chosen. There is no *a priori* reason to suspect that the colonies sampled in 1990 were unlikely to produce zygotes, but given the relatively small number of colonies involved (2–4 per night), the low values could be a random sampling effect. Even if the low values recorded in 1990 are specific to only some colonies, the commonness of low values indicates that on any given night some (and perhaps many) of the colonies on a reef can have almost complete reproductive failure. That failure is driven not by their production of gametes, but by fertilization rates.

Fertilization rates during 1993 and 1994 were among the greatest observed. Some of this effect may be attributable to the shorter incubation times employed during those years, but our comparisons of survival at 12 and 36 h suggest that any such effect would be of smaller magnitude (9%) than the range of variance observed. Furthermore, the difference in incubation regimen cannot explain the presence of near 0% fertilization on two of the nights during August 1994.

Our results also indicate that measures of fertilization based on the ability of water samples to fertilize eggs are generally accurate but probably obscure much of the fine-scale variance in fertilization rates. In our experiments the "fertilization potential" of field-water samples explained only 41% of the variance in the field samples. As Figures 3 and 6 indicate, fertilization potential characterized much of the overall pattern of fertilization rates, but differed from field data in absolute magnitude and exhibited less variance.

Our August 1992 sperm supplementation experiments indicate that the low fertilization rates can unambiguously be attributed to sperm limitation. The consequences of sperm limitation are twofold. First, *Plexaura kuna* has extraordinarily low rates of recruitment in the San Blas (Lasker, 1990, and unpub. data). Although there are probably multiple causes for the recruitment failure, fertilization success places yet another barrier in the cascade of events necessary to generate years with high recruitment. Years with high fertilization rate may not generate high recruitment rates, but high fertilization success is probably a necessary prelude to years with successful recruitment.

The other striking feature of the data is the variability of fertilization rates that can be found at every level of analysis. The source of that variance is ultimately linked to the processes that control the densities of eggs and sperm in the water column, as it is these densities that control *P. kuna* fertilization success. Both the production of gametes and the current regime that dilutes those gametes contribute to the variance in gamete densities and the variance in fertilization success.

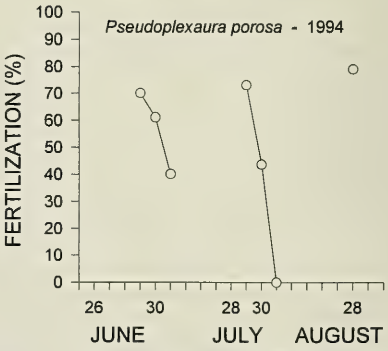

Figure 8. Daily average of proportions of *Pseudoplexaura porosa* eggs fertilized in samples collected from Korbiski Reef on different days.

The pattern of variance in *P. kuna* fertilization rates that we have observed at Korbiski has been driven by variation occurring at a number of different scales. At the level of months and days, there has been the trend throughout our observations for July fertilization success to be greater than June and August. Similarly the first and last day(s) of spawning usually have the lowest rates of fertilization of each month. Much of the day-to-day and month-to-month variation is probably driven by numbers of gametes being released. That and changes in the direction of currents that carry sperm either toward or away from female colonies may explain the daily and monthly pattern. The current direction effect is especially important for *Plexaura kuna* at Korbiski because there are only three male clones present on the reef and current direction can direct their gametes away from our collection sites. This is well illustrated in the 1994 fertilization rates where the decrease in fertilization rate on June 28, 1994 (Fig. 3) can be explained by the current having a different orientation than on either the preceding or following nights.

On a finer scale the variance in fertilization rates that occurs between samples collected within minutes or meters of each other is probably related to fine-scale variance in the release or mixing of sperm as they are released from male colonies. Observations of dye clouds released shortly before spawning events indicate that dye, and presumably sperm, do not diffuse uniformly as they are advected downstream (Lasker and Stewart, 1993). Instead, a highly heterogeneous series of structures are formed that remain surprisingly coherent over a scale of centimeters and meters (*i.e.*, over seconds and minutes depending on current speed). These same types of heterogeneities have also been observed by Moore *et al.* (1992, 1994), in studies of the turbulent diffusion of odor plumes. They have observed heterogeneities on the scale of centimeters and seconds.

The results of the analyses of spawning on Korbiski Reef in the San Blas Islands indicate that the potential for sperm limitation identified in many of the previous studies of broadcast spawning is realized in at least some species. *Plexaura kuna* and *Pseudoplexaura porosa* are common and more importantly represent two extremes in distribution pattern. *P. kuna* is clonal (Lasker, 1984, 1990; Coffroth *et al.*, 1992) and at some sites spreads extensively via fragmentation. Although *P. kuna* at Korbiski is locally very dense, the reef is dominated by two female clones and there are only three male clones on the reef. Furthermore, the male clones are among the smallest clones on the reef. Consequently, there are few males at Korbiski relative to the number of female colonies. In this context *P. kuna* populations can be thought of as groupings of relatively rare, albeit very large, individuals that are often distantly spaced. Under these circumstances the finding of sperm limitation and low fertilization success is not surprising. In contrast, at Korbiski and at many Caribbean localities, *Pseudoplexaura porosa* is common (over 40 large colonies in the 200 m^2 area from which we collected) and has a 1:1 sex ratio. The finding of low fertilization success among the *P. porosa* egg samples suggests that sperm limitation is likely to occur in a wide variety of species and settings.

Our results as well as previous studies (Table I) all demonstrate that fertilization rates are often low and that rates will vary depending on environmental effects such as turbulence and biotic factors such as the numbers of sperm released and spawning behaviors. The effects of these factors operate at two levels, population dynamics and life history evolution. At the population level they may limit population dynamics. Many marine species recruit sporadically or at extremely low levels. One factor that may contribute to these recruitment rates is fertilization. Both Lessios (1988) and Levitan (1988) have speculated that very low population densities following the *Diadema antillarum* pandemic (Lessios *et al.*, 1984) may have reduced fertilization rates and have retarded recovery of *D. antillarum* populations. Similarly, the low fertilization rates observed for *P. kuna* are consistent with the low recruitment rates that we have observed (Lasker, 1990).

Regardless of whether the total number of eggs fertilized limits population growth, variation in fertilization rates has important implications for our understanding of broadcast-spawning species. If the proportion of an individual's gametes which become zygotes predictably varies with respect to that individual's morphology, behavior, or choice of habitats, there will be differential (and perhaps heritable) differences in each genotype's contribution to the next generation. This selection will occur independently of whether the population is actually limited by the supply of zygotes. Thus there should be strong selection pressure for enhanced fertilization success and, to the extent that behavioral, morphologic, or growth strategies can predictably affect fertilization rates, this selection has helped to mold reproductive strategies of broadcast-spawning species (Levitan, 1995; Levitan and Petersen, 1995).

The variance in fertilization success between months and years suggests that predictable reproductive success for many broadcast-spawning species may only occur on a scale of years and decades. This may not be a serious constraint for gorgonians, which are long lived. However, broadcast spawning cannot be expected to be successful unless an individual reproduces over many years, or produces large numbers of gametes and releases them in a fashion that minimizes sperm limitation. This places a premium on both longevity and the production of large numbers of gametes, in other words, long-lived individuals that produce many gametes. This seeming contradiction in strategies can be reconciled if one considers that many of the broadcast-spawning taxa are modular. Modular or clonal growth allows genetic individuals to become extremely long lived and to grow to large spatial size. As a consequence, an individual that lives long and produces prodigious numbers of gametes is created.

It is also clear from the *P. kuna* data that ecologic traits affecting size or spacing also affect fertilization. Clonal propagation, by spreading *P. kuna* clonemates across substantial areas (Coffroth *et al.,* 1992), allows *P. kuna* clones to sample across the spatial variance in fertilization success as well as the temporal variance. The same potential for spatial and temporal variance in fertilization should also exist for wind-pollinated plants. Among many of these species, modular/clonal growth produces large, long-lived structures (*e.g.,* trees or large areal expanses of a grass clone) (Silander, 1985).

Iteroparity across many spawning events increases the odds of spawning at a time favorable to fertilization success, but an alternative strategy is to reduce the likelihood of sperm limitation by increasing the number of sperm released and exhibiting behaviors and gamete traits that enhance the probability of fertilization. Echinoderms provide good examples of these strategies. Work by Babcock and Mundy (1992), Babcock *et al.* (1994), and Benzie *et al.* (1994) has demonstrated that *Acanthaster planci* realizes high fertilization rates by releasing large numbers of sperm during spawning events. Other species such as *Strongylocentrotus franciscanus* and *Diadema antillarum* (prior to the 1983 mass mortality) may rely on high population densities to assure fertilization (Lessios, 1988; Levitan, 1988, 1993; Levitan *et al.,* 1992). Yet other species aggregate and thereby enhance fertilization (Young *et al.,* 1992).

If fertilization rates have played an important role in the life history evolution of broadcast-spawning species, then we should be able to observe tradeoffs between fertilization-enhancing features and the rest of the phenotype that vary with the degree of sperm limitation. For instance, Levitan (1993) has argued that echinoid egg size affects fertilization and that the tradeoff between number and size of eggs is also affected by population density and fertilization success. *P. kuna* and *P. porosa* appear to differ in fertilization rates, and it will be important to determine whether tradeoffs related to sperm limitation can be observed among these and other gorgonians in traits such as sperm longevity, egg size and viability, colony fecundity, distribution, and clonal spread.

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Gastrovascular Flow and Colony Development in Two Colonial Hydroids

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Abstract. Using field-collected *Podocoryne carnea* and *Hydractinia symbiolongicarpus,* ten colonies of each species were produced by sexual crosses and grown from primary polyps to sexual maturity. At comparable stages in the life history, each colony's morphology was measured using digital image analysis, and each colony's gastrovascular flow to three peripheral stolon tips was recorded using video microscopy. Gastrovascular flow to peripheral stolon tips shows very different patterns in the two species. *H. symbiolongicarpus* exhibits maximal flow as a primary polyp. Both the mean and the variance of the flow rate decline by the time of stolonal mat formation. When covering the substratum, gastrovascular flow is low and somewhat more variable than at the time of stolonal mat formation. On the other hand, *P. carnea* exhibits minimal flow as a primary polyp. At subsequent stages, flow increases monotonically. Thus at the primary polyp stage, *H. symbiolongicarpus* exhibits a greater rate of flow to peripheral stolon tips than *P. carnea.* The reverse is true at subsequent stages. In general, these results support the hypothesis that higher rates of gastrovascular flow produce runner-like colonies, while lower rates of flow produce sheet-like colonies.

Introduction

In colonial hydroids, two kinds of pattern-forming events can be recognized, those events that contribute to patterning in single polyps and those events that contribute to colony-wide patterns in the arrangement of polyps and stolons. While the former class of events are increasingly well understood (*e.g.,* Shenk *et al.,* 1993; Shenk, Gee, *et al.,* 1993), the latter events remain obscure. Further, colony-wide patterns are not likely to be a simple "scaling up" of polyp-level events. For instance, there is no polyp-level analog to within-colony (*i.e.,* between-polyp) gastrovascular flow; nevertheless, increasing amounts of evidence suggest that such flow mediates important aspects of colony pattern and life history, as for instance in the hydractiniid hydroids (Blackstone and Buss, 1991, 1992, 1993). Curiously, despite the potential importance to pattern formation, within-colony gastrovascular flow remains poorly characterized, *e.g.,* how does such flow vary as a colony develops from a primary polyp to sexual maturity?

This question is particularly relevant to the hydractiniid hydroids, in which species of *Hydractinia* and *Podocoryne* generally exhibit contrasting suites of morphological and life history traits ("sheets" *versus* "runners"), and in this way exhibit patterns characteristic of many colonial animals (Buss and Blackstone, 1991). Experimental studies of *Hydractinia symbiolongicarpus* and *Podocoryne carnea* suggest that gastrovascular flow may trigger changes in the timing of colony development and thus mediate these morphological and life history differences (Blackstone and Buss, 1992, 1993). This work has led to hypotheses concerning the relationship between gastrovascular flow and colony development, *e.g.,* that flow to peripheral stolons in *H. symbiolongicarpus* will decrease relative to *P. carnea* as colony development proceeds and as the former assumes a sheet-like morphology relative to the latter. Nevertheless, the actual relationship between gastrovascular flow and major features of colony development remains unclear (*e.g.,* does gastrovascular flow diminish when a sheet-like morphology first becomes apparent in *H. symbiolongicarpus?*).

One difficulty in addressing these questions is that clonal repeatability studies of field-collected colonies cannot be used. Such experiments are common with hydractiniid hydroids and are informative because they provide a measure of within-colony variance (*e.g.,* McFadden *et al.,* 1984; Buss and Grosberg, 1990; Blackstone and Buss, 1991). Nevertheless, because field-collected colonies

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have completed key aspects of colony development (in particular, the primary polyp stage and the formation of the stolonal mat), they cannot be used to investigate colony development definitively. Rather, to investigate gastrovascular flow during colony development, it is necessary to carry out sexual crosses of field-collected colonies and to observe the complete development of the offspring colonies.

This study compared 10 full-sib colonies of each of two species of hydractiniid hydroid (*P. carnea* and *H. symbiolongicarpus*). The gastrovascular flow and the morphology of these colonies were compared at three stages in their life history (stage 1: as primary polyps, stage 2: at the time of stolonal mat formation in *H. symbiolongicarpus,* and stage 3: upon attaining large colony size and nearly covering the substratum). While the issue of developmental equivalence is complex (*e.g.,* see Blackstone, 1987), the goal here is to compare these colonies as early in development as possible (as primary polyps), as late in development as possible (when nearly covering the substratum), and as near to a critical developmental landmark in *H. symbiolongicarpus* as possible (see further discussion in Methods). Because these are small samples of related individuals, it is crucial to assess the extent to which the observed variation in these full-sib families mirrors the range of variation in the wild populations. Consequently, at a comparable stage of colony development (stage 3), the results from the full-sib individuals were related to results from field-collected colonies. The data indicate that gastrovascular flow in these two species of hydractiniid hydroid follows a very different time course during development, and that these differences in flow correlate with the observed differences between these species in morphology and life history.

Materials and Methods

Production of colonies and culture conditions

Colonies on hermit crab shells were collected from the Yale Peabody Museum Field Station in Connecticut. Two mature *H. symbiolongicarpus* individuals were mated using standard methods (*e.g.,* Spindler and Müller, 1972; Weis and Buss, 1987; Blackstone and Buss, 1991). *P. carnea* individuals are more difficult to breed because their life cycles include a medusa stage. Medusae were obtained from a male and female colony and were cultured together until sexually mature with feedings of brine shrimp three times per week. At 20.5°C, medusae reached sexual maturity in less than a week and produced viable gametes for over a month. Competent larvae of both species were induced to metamorphose by means of ionic imbalance (53 m*M* CsCl solution, see Spindler and Müller, 1972; Weis and Buss, 1987; Blackstone and Buss, 1991), and metamorphosing larvae were deposited on 15 mm diameter round coverslips. Colonies were effectively confined to one side of the coverslips by cutting back encrusting stolons from the reverse side on a daily basis. Coverslips were suspended in floating racks and grown in 120-l aquaria containing Reef Crystals artificial seawater (salinity = 35‰) with temperature control to 20.5 ± 0.5°C, undergravel filtration, and 50% water changes weekly. Ammonia, nitrites, and nitrates were maintained below detectable levels (Aquarium Systems test kits). Colonies were fed to repletion with brine shrimp nauplii three days per week. Analysis has shown that, with similar culture conditions, "random" statistical effects (*e.g.,* time effects, tank effects, rack effects, see Sokal and Rohlf, 1981) are negligible (Blackstone and Buss, 1991).

Colony development

Colony development in both taxa begins with the metamorphosis of the planula larva into a primary polyp. Runner-like stolons extend from the primary polyp. Stolons encase fluid-filled canals that are continuous with the gastrovascular cavity of the polyp. In cross-section, stolons consist of a fluid-filled lumen encased by endoderm, ectoderm, and a rigid periderm. Gastrovascular fluid circulates in the lumen of the stolons and carries food and possibly other metabolites from the feeding polyp to other parts of the colony; contractions of the muscular polyp propel the gastrovascular fluid (Schierwater *et al.,* 1992). As the lumen fills and empties in response to contractions of the polyps, the endodermal and ectodermal tissue layers of the stolon expand and contract as well (see further discussion below). The rigid periderm, however, remains fixed and sets the maximum diameter for stolonal expansion. Primary polyps in *P. carnea* and *H. symbiolongicarpus* appear indistinguishable, except for the differences in stolon widths (see results). From a primary polyp, *P. carnea* develops into a colony by lineal extension of the stolons, initiation of new stolonal tips, and iteration of feeding polyps on the stolons. *P. carnea* colonies thus form a loose network of polyps and stolons typical of many runner-like forms (Fig. 1). Once the available substratum is covered, *P. carnea* colonies increase polyp and stolonal tip formation, producing a more closely knit network of stolons and ultimately initiating the sexual (medusoid) phase of the life cycle. While the mature colony morphology of *P. carnea* is somewhat sheet-like, the ectodermal cell layers of the stolons do not fuse. In contrast, soon after primary polyp formation in *H. symbiolongicarpus,* stolons fuse to form a continuous stolonal mat, a closely knit complex of anastomosing stolons capped by a continuous layer of ectoderm, which shows sheet-like growth, and from which extend relatively few peripheral stolons (Fig. 1). At sexual maturity, *H. symbiolongicarpus* colonies completely cover the substratum with a spiny stolonal

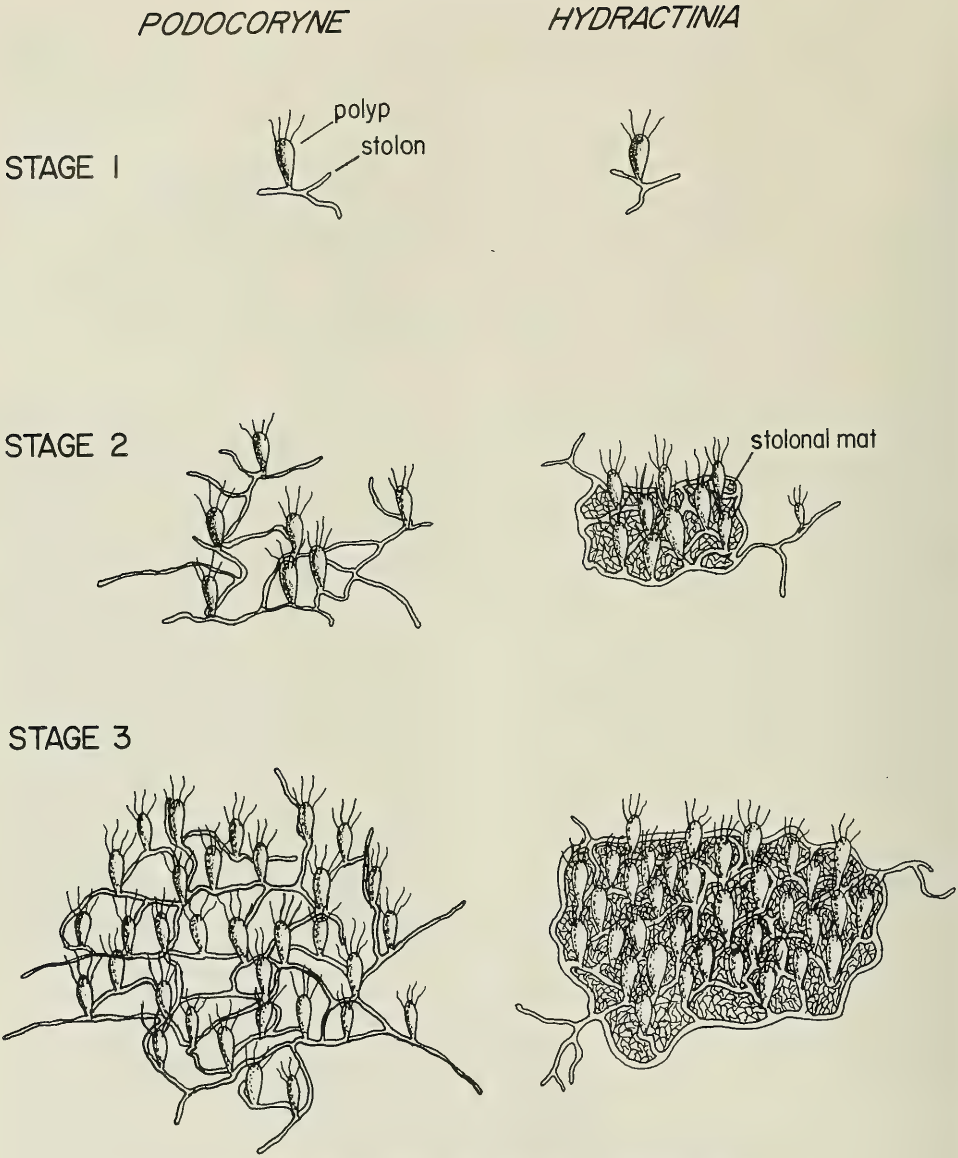
PODOCORYNE
HYDRACTINIA
STAGE 1
polyp
stolon
STAGE 2
stolonal mat
STAGE 3

Figure 1. Schemata illustrating the stages of colony development in hydractiniid hydroids. Both *Podocoryne carnea* and *Hydractinia symbiolongicarpus* initiate colony development as primary polyps (stage 1). Stage 2 is defined as the time that *H. symbiolongicarpus* colonies first develop a stolonal mat (the basal area with many branching and anastomosing stolons). At stage 3, colonies are nearly covering the substratum.

mat. Mature *P. carnea* exhibit a juvenilized colony morphology, developing to sexual maturity without forming a true stolonal mat, while *H. symbiolongicarpus* forms a stolonal mat early in colony development, well before sexual maturity (Blackstone and Buss, 1991, 1993).

This study compares colony morphology and within-colony flow primarily at three stages of development (Fig. 1). In stage 1, both species are primary polyps and have not yet formed colonies (hence morphology was not measured). Flow to three stolon tips was measured 3–5 days after metamorphosis; only primary polyps with three or more stolons were included in the study. Stage 2 was defined in *H. symbiolongicarpus* as the day immediately after stolonal mat formation became visible in a colony. Stolonal mat formation varied both in terms of colony size (*e.g.*, range of 10–50 polyps per colony, mean ± SE, 20.6 ± 3.9; range of 2.65–37.93 mm^2 total area, mean ± SE, 9.67 ± 3.28) and in terms of chronological time (range of 11–22 days after metamorphosis, mean ± SE, 16.5 ± 1.1). *P. carnea* does not form a stolonal mat, so flow and colony morphology were measured at a similar colony size and chronological time (range of 3.68–21.36 mm^2 total area, mean ± SE, 7.80 ± 1.62, this is not significantly different from *H. symbiolongicarpus,* $F = 0.26$, df = 1, 18, $P > 0.60$; range of 15–22 days after metamorphosis, mean ± SE, 18.7 ± 1.0, this is not significantly different from *H. symbiolongicarpus,* $F = 2.26$, df = 1, 18, $P > 0.15$). Morphology and gastrovascular flow were also measured at stage 3, which was defined as shortly before the colony covered the substratum (~177 mm^2). Morphological measures were also taken at the time that each colony covered roughly half of the substratum, and at the time of sexual maturity (peripheral flow cannot be measured at this time because there are no peripheral stolon tips). Because the timing of stolonal mat formation in *H. symbiolongicarpus* had to be measured before the stage 2 measures of *P. carnea* could be done, the *H. symbiolongicarpus* colonies were grown and measured first. This experimental design introduces a potentially confounding time effect. Nevertheless, using similar culture conditions, previous work has not detected a significant time effect (Blackstone and Buss, 1991). Further, the stage 3 measures of flow were explicitly compared to measures from experiments in which between-species comparisons were randomized with respect to time, and in both cases the same between-species effect is apparent (see results).

Colony image analysis and morphometrics

Colonies were measured using image analysis technology (see Rohlf and Bookstein, 1990). Briefly, a high-resolution MTI CCD-72 camera attached to a macro lens was used to project each colony onto a color monitor interfaced with a PC compatible microcomputer (Pentium/90 MHz CPU, no floating-point defect, 16 Mb RAM) equipped with an Overlay Frame Grabber board (640 × 480 pixels with 12 bit depth per pixel). Using OPTIMAS software, background-subtracted images of the colonies were acquired with illumination appropriate to produce three distinct luminance thresholds: the polyps (lightest), the stolons (intermediate), and the empty coverslip (darkest). Using these thresholds, the software identified and measured the areas of the empty coverslip and of the individual polyps (Blackstone and Buss, 1992). Classification macros were used to identify and exclude coverslip areas outside the edge of the colony. The total colony area and perimeter were also measured. Data files were analyzed using PC-SAS software. For each colony at each measurement time, the total area of polyps and the total area of empty coverslip enclosed within the colony were expressed as a fraction of the total area (note that the total area of stolons and of stolonal mat, if present, can be calculated as 1 minus this combined fraction). Morphology was further measured with several metrics that usefully describe colony development (see Blackstone and Buss, 1991, 1992, 1993), including (1) the colony's total area of polyps divided by the mean size of the areas of empty coverslip enclosed within the colony's stolons, and (2) the total colony perimeter divided by the square root of the total colony area. Categorical statistics were used to compare the numbers of colonies of each species which covered the surface with a perfect sheet (*i.e.,* areas of empty coverslip enclosed within the colony's stolons = 0, and hence mean inner area = 0).

Video microscopic measures of peripheral gastrovascular flow

To characterize gastrovascular flow, ideally the volume and velocity of the fluid in a stolon should be measured. In fact, it is a non-trivial task to accurately measure the interior of a living specimen in a water medium in a non-invasive manner in real time. Of the several potentially applicable techniques, the most promising is optical slicing (L. Buss, pers. comm.). Nevertheless, the appropriate instrument for this application has yet to be developed.

At present, video microscopy provides an suitable alternative. Gastrovascular flow reaches a maximum 2–8 h after feeding (Schierwater *et al.,* 1992); all of these studies were carried out 3–5 hours after feeding. The colony was placed in a flow-through chamber with a #1 coverslip base (Warner Instruments). The temperature of the in-flowing seawater was adjusted with a thermoelectric device to maintain a constant chamber temperature (20.5 ± 0.3°C; chamber temperature was monitored with a YSI cuvette thermometer with a flexible probe). Colonies were viewed on an inverted light microscope (Zeiss Axiovert 135), with a 40× Achroplane objective in Differential Interference

Contrast. Using the MTI CCD camera, three primary stolon tips from each colony were videotaped for 10 min each.

Gastrovascular flow must be reversed in each distal, "dead-end" tip. Stolon tips fill as fluid enters; the velocity of the fluid then decreases to zero. Tips then empty, and the fluid velocity again decreases to zero. In the region of the stolon immediately behind the tip, the difference between the width of the stolon lumen when it is at a maximum (and fluid velocity is zero) and when it is at a minimum (and velocity is again zero) provides a measure of the rate of gastrovascular flow, if this difference is measured over time (Fig. 2). These width or diameter measures are taken at the base of the lumen (Fig. 2). With the image analysis system connected to the VCR, the diameter of the stolon lumen was measured at a point ~250 μm behind the tip itself. In this region of the stolon, gastrovascular fluid velocity goes to zero as the lumen diameter approaches its maximum and minimum (thus velocity itself need not be measured). Lumen diameter was measured when the stolon was full and when it was empty for three consecutive, but non-overlapping, cycles (in a few cases, only two cycles occurred in the 10-min interval). For each cycle, the net amplitude, that is, the difference between the maximum and minimum lumen diameters, was calculated. Periderm-to-periderm total stolon diameter (which is invarient throughout the contraction cycle) was also measured, and net amplitude was usually divided by the total stolonal diameter. The period (in seconds) of each cycle was also measured.

The interpretation of these measures in terms of the volume of gastrovascular flow depends on the cross-sectional shape of the stolonal lumen, and on how this shape changes during the contraction cycle. While it is not possible to visualize this shape clearly in the colonies used in these experiments, stock colonies with stolons growing on the edge of the coverslip allow this shape to be observed. In general, peripheral stolon tips tend to be broad and flattened. When the stolon is filled, the lumen roughly resembles half an ellipse in cross-sectional shape (Fig. 2; the base of the half-ellipse corresponds to the measured lumen diameter). The exact mechanisms of stolonal contractions are complex, depending in part on a unique endodermal organelle (Schierwater *et al.*, 1992), and in part on a general contraction of the stolonal tissues. The contraction of tissues, however, does not occur uniformly. Tissues lying along the broad, flattened base of the stolon contract very little. Rather, it is the tissues that make up the sides and "roof" of the lumen that collapse inward. In many cycles, the contracted lumen forms a relatively deep and narrow central channel (Fig. 2).

Given the complex behavior of the stolon tip, and the absence of instrumentation to precisely measure stolon tip volumes, the linear change in the basal diameter of the stolonal lumen was used to estimate the change in volume. Derived measures assuming a constant, circular cross-section (*e.g.*, maximum lumen radius squared minus minimum lumen radius squared) will overestimate the change in volume, and such measures were not generally used. In fact, while analyses focused on the linear, measured variables, analyses based on the squared measures provide similar results (see Results).

An additional concern is the slight change in stolonal volume associated with stolon tip growth pulsations (see Beloussov *et al.*, 1989). In hydractiniid hydroids, these pulsations are small, and, from the point that stolonal

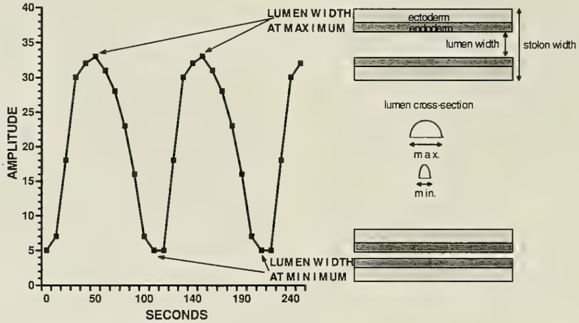

Figure 2. The contraction and expansion of the stolon lumen as it fills and empties in response to gastrovascular flow. Amplitude for each cycle corresponds to the diameter of the lumen. Schemata show transverse- and cross-sections of the stolon tip with the lumen at maximal and minimal width. In the region of the stolon immediately behind the tip, the difference between the width of the stolon lumen when it is at a maximum (and fluid velocity is zero) and when it is at a minimum (and velocity is again zero) provides a measure of the rate of gastrovascular flow, if this difference is measured over time.

diameter was measured, they amount to less than 1% of the length of the stolon tip. Further, these tip pulsations occur in both of the study species, and are thus not likely to introduce a significant between-species effect.

Finally, it is a concern as to whether measures of lumen diameter should be adjusted for differences in the size of the stolon. Given that the primary function of the lumen is food transport, and that larger stolons will require more food, biologically it is reasonable to adjust the amount of flow for the amount of tissue requiring food. Generally, therefore, measures of the stolonal lumen are adjusted for total stolonal diameter, although, in fact, unadjusted measures provide similar results (see Results).

In general, it should be recognized that in this experimental system maximum lumen diameter and wall thickness cannot be measured independent of the flow. The stolon generally closes to a low minimum diameter during outflow. Fluid inflow then inflates it; in the stolon tip the amount of inflation is proportional to the maximum lumen diameter. Thus the maximum lumen diameter will necessarily be similar to the amount of flow, and it would be entirely artifactual to correlate the amount of flow to the maximum lumen diameter. Further, while it is possible to estimate wall thickness (periderm-to-periderm stolonal diameter minus maximum lumen diameter), this measure is again confounded with flow; walls may seem thin because a large inflow compressed the ectoderm more completely against the periderm.

Statistical analysis thus focused on two outcomes: the net amplitude (maximum minus minimum) of the change in the stolon lumen diameter and the period for each cycle (Fig. 2). When lumen amplitude was adjusted for stolon width, it is termed the "relative amplitude." Particularly for illustrative purposes, amplitude and period were combined by division into a rate of expansion or contraction of the stolon lumen, again adjusted for the width of the stolon (μm of lumen diameter per total μm of stolon width per second). Biologically, this rate measure illuminates the "rate of supply" of food to the tissues of the stolon tip. All these variables generally meet the assumptions of parametric statistics (see Sokal and Rohlf, 1981). Nested univariate and multivariate analysis of variance was used with cycles nested within stolons, stolons nested within colonies, and colonies nested within species. For a few stolons, only two cycles occurred in the 10-min interval; this produced a slightly unbalanced design. Examination of the coefficients of the variance components suggests that the F-ratios calculated are thus slightly conservative (*i.e.,* if the null hypothesis is correct, the listed *P* values slightly overestimate the probability that the observed data could have arisen by chance alone). The analysis was done separately for each developmental stage. While developmental stage could be included as a predictor in the statistical model, it is very difficult to classify this variable as a fixed or a random effect (see Sokal and Rohlf, 1981). It is likely that developmental stage is partly a fixed effect (*e.g.,* for primary polyps, this developmental stage is seemingly common to all colonies of both species), and partly a random effect (*e.g.,* for stolonal mat formation, this developmental stage may be common to all *H. symbiolongicarpus* colonies, but it is clearly not common to the *P. carnea* colonies). Carrying out the ANOVAs and MANOVAs separately for each stage is a solution to this difficulty. Comparisons between individuals of the same species at different developmental stages was done with paired comparison *t*-tests (Sokal and Rohlf, 1981).

In the course of these analyses, numerous statistical tests were carried out. Since only *a priori* hypotheses were tested, alpha levels were not adjusted for multiple comparisons. In general, it is perhaps best to view the results in totality, rather than to focus on any particular P value (see Rothman, 1986; Goodman and Royall, 1988; Goodman, 1993).

Additional morphological and life history comparisons

Previous observations have suggested numerous morphological and life history differences between *P. carnea* and *H. symbiolongicarpus,* consistent with characterizing the former as a "runner" and the latter as a "sheet." Differences in growth rate have been established (Blackstone and Buss, 1991), and the medusa and swimming planula of *P. carnea* clearly suggest greater dispersal abilities than in *H. symbiolongicarpus,* which lack medusa and have slow-crawling planula. Nevertheless, no data have been obtained on the tendency of *H. symbiolongicarpus* to develop greater quantities of spines (this species is part of the "*echinata*" complex, see Buss and Yund, 1989) or on the relative timing of reproduction in the two species.

The timing of reproduction in each species was compared by counting the days from metamorphosis until mature gonozoids were first observed for each colony, and these data were analyzed with an ANOVA of the log-transformed data. Measures of spine production are complicated because in the abrasion-free laboratory environment, spines are not formed. However, dark-colored deposits of the material used to form spines (likely a chitin-protein complex) can be seen in the stolonal mat of laboratory colonies of *H. symbiolongicarpus.* This contrasts to the generally pigment-free appearance of both *H. symbiolongicarpus* and *P. carnea.* To very roughly gauge differences in the amounts of these dark pigments at sexual maturity, each colony was illuminated with an identical intensity of visible light and the transmission of this light through the colony was examined densitometrically. Using the 40× Achroplane objective of the Zeiss Axiovert microscope, 5 replicate measures were obtained for each of the 10 colonies of each species. The five measured areas of each colony were selected haphazardly near the center of each colony; however, if such an area encompassed a polyp or an empty area of coverslip, another area was selected before the densitometric measure was taken. Using the OPTIMAS software, the amount of light transmitted was taken as the mean value of the luminance histogram for each area. These means were analyzed with a nested ANOVA in which the between-species effect was tested against the colony-within-species effect.

Mechanisms relating flow to colony development

If stolonal gastrovascular flow is a key aspect of stolon (and hence colony) development, such flow should initiate at the time of the appearance of stolons. At room temperature, this occurs roughly 20 h after metamorphosis begins, that is, prior to the actual formation of a primary polyp (see discussion in Weis and Buss, 1987). Metamorphosing larvae at this time are not firmly attached to the substratum and cannot be moved. Further, while such "pre-primary" polyps may have a mouth, they do not have fully developed tentacles and are incapable of feeding. To observe gastrovascular flow in such polyps, 10 larvae of the same *H. symbiolongicarpus* parents as used above were metamorphosed directly in the microscope chamber at room temperature (19–21.5°C). After 20 h, the water in the chamber was replaced and the chamber temperature was adjusted to 20.5 ± 0.3°C. Polyps were filmed as described above, except one stolon rudiment was filmed per polyp (at the time of filming, most of these polyps had only one such rudiment). These data are not directly comparable to the other studies of flow, since these polyps were unfed. Nevertheless, it is of interest whether flow at this time is non-zero.

Plausibly, gastrovascular flow could influence colony morphology by directly stimulating the growth of stolon tips (*e.g.*, by a greater availability of food). If this is the case, a greater rate of stolonal contraction and expansion would be expected to produce a greater rate of distal tip growth (Wyttenbach, 1973). To examine this hypothesis, flow to two stolonal tips was measured for three clonal replicates of each of nine field-collected colonies of both *H. symbiolongicarpus* and *P. carnea* (see Blackstone and Buss, 1991, 1992, for a more detailed description of the methodology). Immediately following this measure of the flow, the microscope objective was shifted to the tip of the stolon and growth for a 15-min interval was measured. For each stolon, the mean rate of flow was then correlated to the amount of tip growth using parametric statistics. The between-species difference in tip growth was tested by an ANOVA with the colony-within-species effect as a error term.

Results

Colony morphometrics

A representative sample of the colony images is available on the World Wide Web (http://www.bios.niu.edu/eande/Blackstone.html). Colonies were measured at the time of stolonal mat formation in *H. symbiolongicarpus* (stage 2), at the time of covering half the substratum, at the time of nearly fully covering the substratum (stage 3), and at the time of initiating sexual reproduction (in *H. symbiolongicarpus*) or the sexual phase of the life cycle (in *P. carnea*). With the exception of the last stage (when many of the measured polyps were gonozoids), colonies of *H. symbiolongicarpus* generally have a greater total area of polyps and a lesser total area of empty space enclosed within the colony (Fig. 3). Compared to *P. carnea*, at the time of stolonal mat formation (stage 2) colonies of *H. symbiolongicarpus* have a significantly greater polyp area (mean ± SE for *H. symbiolongicarpus*, 0.16 ± 0.02; for *P. carnea*, 0.05 ± 0.009; F = 8.36, df = 1, 18, $P < 0.01$) and a significantly smaller area of empty space (mean ± SE for *H. symbiolongicarpus*, 0.17 ± 0.04; for *P. carnea*, 0.28 ± 0.03; F = 5.41, df = 1, 18, $P < 0.04$). Similarly, at the time of covering half the substratum colonies of *H. symbiolongicarpus* have a significantly greater polyp area (mean ± SE for *H. symbiolongicarpus*, 0.08 ± 0.008; for *P. carnea*, 0.05 ± 0.008; F = 8.58, df = 1, 18, $P < 0.01$) and a significantly smaller area of empty space (mean ± SE for *H. symbiolongicarpus*, 0.14 ± 0.04; for *P. carnea*, 0.48 ± 0.04; F = 33, df = 1, 18, $P \ll 0.001$). At the time of nearly fully covering the substratum (stage 3), differences in polyp area are no longer apparent (mean ± SE for *H. symbiolongicarpus*, 0.13 ± 0.02; for *P. carnea*, 0.12 ± 0.02; F = 0.12, df = 1, 18, $P > 0.7$), while differences in area of empty space persist (mean ± SE for *H. symbiolongicarpus*, 0.11 ± 0.04; for *P. carnea*, 0.28 ± 0.03; F = 10.85, df = 1, 18, $P < 0.01$). At the time of initiating sexual reproduction or the sexual phase of the life cycle, measures of polyp area are heavily influenced by the numbers of reproductive polyps. *P. carnea* colonies have a greater total area of polyps, and this difference is nearly significant (mean ± SE for *H. symbiolongicarpus*, 0.25 ± 0.03; for *P. carnea*, 0.36 ± 0.04; F = 4.41, df = 1, 18, $P < 0.06$). Also at this time the area of empty space in all *H. symbiolongicarpus* colonies is 0, and the area of empty space in *P. carnea* colonies is small but non-zero (mean ± SE, 0.04 ± 0.01). Significantly more *H. symbiolongicarpus* colonies formed perfect sheets (*i.e.*, number and mean of inner areas = 0) than *P. carnea* colonies (number of sheets: number of non-sheets; for *H. symbiolongicarpus* 10: 0, for *P. carnea* 1: 9; Fisher's exact test, $P < 0.001$).

Metrics used previously (*e.g.*, Blackstone and Buss, 1991, 1992) illustrate the same differences. Compared to *P. carnea*, at the time of stolonal mat formation (stage 2)

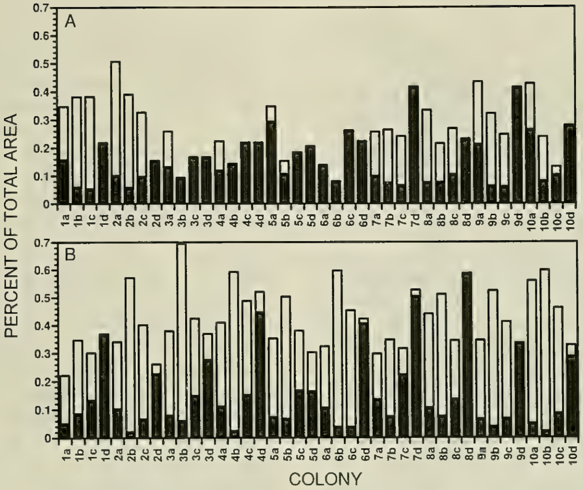

Figure 3. Each bar represents the polyp area divided by the total area of the colony (filled portion) and the area of empty coverslip enclosed within the colony divided by the total area of the colony (open portion) for *H. symbiolongicarpus* (A) and *P. carnea* (B). Numbers correspond to individual colonies; letters correspond to the times at which colonies were measured. Each colony was measured at the time of stolonal mat formation in *H. symbiolongicarpus* (a = stage 2), at the time of covering half the substratum (b), at the time of nearly fully covering the substratum (c = stage 3), and at the time of initiating sexual reproduction (in *H. symbiolongicarpus*) or the sexual phase of the life cycle (in *P. carnea*) (d). Note that only one *P. carnea* (colony 8) formed a perfect sheet; the other *P. carnea* always maintained small amounts of empty areas.

colonies of *H. symbiolongicarpus* are significantly more circular in shape (perimeter/$\sqrt{\text{area}}$, mean ± SE for *H. symbiolongicarpus,* 14.50 ± 2.04; for *P. carnea,* 22.39 ± 1.35; $F = 10.43$, $df = 1, 18$, $P < 0.01$) and have more polyps and smaller inner areas (polyp area/mean inner area, mean ± SE for *H. symbiolongicarpus,* 23.73 ± 4.63; for *P. carnea,* 5.70 ± 1.80; $F = 14.3$, $df = 1, 17$, $P < 0.001$; note that one of the *H. symbiolongicarpus* had to be excluded from this analysis because it had already formed a perfect sheet, hence polyp area/mean inner area $= \infty$). At the time of nearly covering the surface (stage 3), there was no statistically significant between-species difference for either of these measures. Note that at this time: (1) shape could not be meaningfully measured, since each colony had reached the edge of the cover slip throughout most of its area, and thus all colony shapes were approaching circularity, and (2) most of the *H. symbiolongicarpus* had formed a perfect sheet and had to be excluded from the polyp area/mean inner area analysis.

Video microscopic measures of peripheral gastrovascular flow

Gastrovascular flow in *H. symbiolongicarpus* and *P. carnea* differs significantly at each stage of development (Fig. 4). As primary polyps, *H. symbiolongicarpus* has a faster rate of expansion and contraction of the stolon lumen (Fig. 4a; testing the between-species effect against the colony-within-species effect; $F = 16.51$, $df = 1, 18$, $P < 0.001$). This faster rate in *H. symbiolongicarpus* derives from a greater, but not significantly so, relative amplitude ($F = 2.51$, $df = 1, 18$, $P = 0.13$) and a significantly shorter period ($F = 11.41$, $df = 1, 18$, $P < 0.004$). These two outcomes can be combined in a MANOVA, which also shows a significant between-species effect (again, using the colony-within-species effect as the error variance; $F = 8.69$, $df = 2, 17$, $P < 0.003$). This MANOVA indicates that the difference between the species entails a difference in the relationship between the relative amplitude and the period of the stolonal contraction cycle, which in this case is analogous to the rate difference depicted in Figure 4a.

At the time that the *H. symbiolongicarpus* colonies form a stolonal mat, gastrovascular flow has changed dramatically. Now, *P. carnea's* stolon lumen has a faster rate of expansion and contraction (Fig. 4b; $F = 10.43$, $df = 1, 18$, $P < 0.005$). This difference in rate derives from a now much greater relative amplitude in *P. carnea* ($F = 68.99$, $df = 1, 18$, $P \ll 0.001$), while *H. symbiolongicarpus* still retains a slightly, but significantly, shorter period (F =

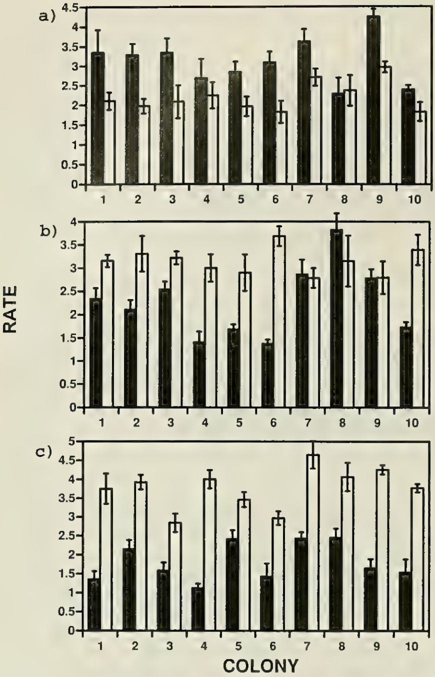

Figure 4. Means and standard errors of rates times 10^3 of lumen expansion and contraction (μm of lumen diameter per total μm of stolon width per second) for 10 colonies of each of *P. carnea* (open bars) and *H. symbiolongicarpus* (filled bars) at three stages of colony development (a = stage 1, b = stage 2, c = stage 3). Colonies of the two species are paired arbitrarily.

14.63, df = 1, 18, $P < 0.002$). In this case, the significant MANOVA is less informative, because it merely indicates that both relative amplitudes and periods are significantly greater in *P. carnea.*

As colonies achieve larger size and approach covering of the substratum, the faster rate of expansion and contraction of the stolon lumen in *P. carnea* is more pronounced (Fig. 4c; F = 68.5, df = 1, 18, $P \ll 0.001$). The much greater relative amplitude of *P. carnea* remains (F = 92.81, df = 1, 18, $P \ll 0.001$), and now there is no difference in the cycle periods (F = 1.47, df = 1, 18, $P >$ 0.24). The significant MANOVA (F = 43.89, df = 2, 17, $P \ll 0.001$) is analogous to the rate difference seen in Figure 4c.

It is instructive to investigate how the developmental changes in the rate of stolonal expansion and contraction are achieved in each species. In *H. symbiolongicarpus,* mean rate significantly decreases between stage 1 and 2 (paired comparison *t*-test of mean rates for each colony, $t = -2.96$, $P < 0.02$) and between stage 1 and 3 (t = -5.17, $P < 0.001$). This decrease in rate is brought about by a decrease in the mean relative amplitude between stage 1 and 2 (t = -3.03, $P < 0.02$) and stage 1 and 3 (t = -5.02, $P < 0.001$) and a concomitant small increase in the mean period between stage 1 and 2 (non-significantly; $t = 1.23$, $P > 0.25$) and between stage 1 and 3 (t = 2.40, $P < 0.04$). Interestingly, the variability of the stolonal contraction rate (including both within- and between-stolon variance) significantly decreases between stage 1 and 2 (paired comparison *t*-test of the standard deviations of rates for each colony, t = -3.41, $P < 0.008$), but not between stage 1 and 3 ($t = -1.46$, $P > 0.15$). Thus the rate of stolonal expansion and contraction in *H. symbiolongicarpus* shows the least variability at the time of stolonal mat formation (Fig. 5; see discussion in Blackstone and Buss, 1993), although the actual mean rate is lowest at stage 3. The change in the variability of rate is brought about largely by changes in the variability of relative amplitude, the standard deviation of which decreases significantly between stage 1 and 2 (t = -2.47, $P < 0.04$) but not between stage 1 and 3 ($t = -1.09$, $P > 0.30$). Cycle period shows no change in variability (between stage 1 and 2, t = -0.14, $P > 0.85$; between stage 1 and 3, $t = -0.02$, $P > 0.95$).

In *P. carnea,* mean rate significantly increases between stage 1 and 2 (paired comparison *t*-test of mean rates for each colony, t = 4.72, $P < 0.001$) and between stage 1 and 3 ($t = 12.5$, $P \ll 0.001$). This increase in rate is brought about by an increase in the mean relative amplitude between stage 1 and 2 ($t = 5.75$, $P < 0.001$) and stage 1 and 3 ($t = 8.12$, $P \ll 0.001$) and no change in the mean period between stage 1 and 2 (t = 1.06, $P > 0.3$) and between stage 1 and 3 (t = -0.59, $P > 0.5$). The variability of both

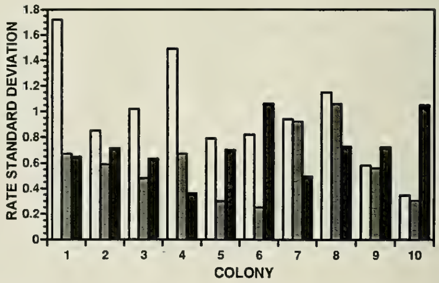

Figure 5. The standard deviations of the rate times 10^3 of lumen expansion and contraction (μm of lumen diameter per total μm of stolon width per second) for 10 colonies of *H. symbiolongicarpus* at 3 stages of development (open bars = stage 1, hatched bars = stage 2, filled bars = stage 3).

rate and period do not change in *P. carnea,* although there is a decrease in the variability of relative amplitude between stage 1 and 3 (paired comparison *t*-test of the standard deviations of relative amplitudes for each colony, $t = -3.88$, $P < 0.004$). Because both rate and relative amplitude are maximal in stage 3, it may be that a constraint is placed on the expansion of the stolonal lumen by the rigid periderm, thus limiting the variability of the amplitude and relative amplitude.

In general, the results presented here agree with those reported elsewhere. Other investigators have also reported high rates of expansion and contraction of the stolonal lumen in *H. symbiolongicarpus* primary polyps (*e.g.,* Buss and Vaisnys, 1993). Further, nine field-collected colonies of each of *H. symbiolongicarpus* and *P. carnea* show similar between-species differences (Blackstone and Buss, 1992). Using replicates of clonally explanted colonies grown to a developmental stage comparable to stage 3, between-species differences in the rate of expansion and contraction of the stolon lumen are very similar to those reported here (see Fig. 6; using the colony-within-species effect as the error variance; F = 33.77, df = 1, 16, $P \ll 0.001$). Note that these studies were carried out under generally similar conditions except (1) colonies from the two species were paired in time so that time effects were explicitly controlled for, and (2) colonies were grown and filmed at 16°C. Given (2) it is unsurprising that the rates of flow in Figure 6 are generally less than those in Figure 4c, but it is nevertheless notable that the between-species effect remains pronounced in both cases.

The measures of periderm-to-periderm stolon width show a consistent between-species effect throughout colony development (Fig. 7). At all stages, *H. symbiolongicarpus* have wider stolons than *P. carnea* (testing the between-species effect against the colony-within-species effect; for stage 1, F = 26.9, df = 1, 18, $P \ll 0.001$; for stage 2, F = 168.05, df = 1, 18, $P \ll 0.001$; for stage 3, F = 9.24, df = 1, 18, $P < 0.01$). Inasmuch as relative amplitudes are adjusted for stolon width, the between-species differences in relative amplitude and in rate for stage 2 and 3 (Fig. 4b, c) may be the result of a similar absolute amplitude of stolonal contraction and the observed width difference. In fact, the actual pattern is more complex. In stage 1, *H. symbiolongicarpus* has a greater unadjusted amplitude of stolon contraction and expansion (F = 16.58, df = 1, 18, $P < 0.001$). In stage 2, *P. carnea* has a greater amplitude, but this difference is non-significant (F = 3.49, df = 1, 18, $P > 0.05$). In stage 3, however, *P. carnea* has a much greater unadjusted contraction amplitude (F = 90.45, df = 1, 18, $P \ll 0.001$), despite narrower total stolon widths. In stage 3, *P. carnea* is thus circulating a greater amount of gastrovascular fluid to its stolon tips, both absolutely and relatively. The observed difference in the rate of stolonal contraction and expansion is not strictly a function of stolonal width differences.

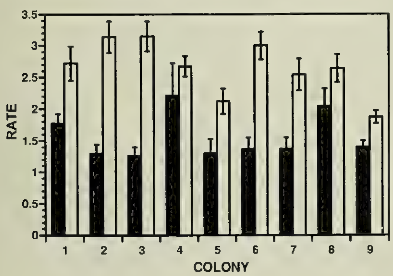

Figure 6. Means and standard errors of rates times 10^3 of lumen expansion and contraction (µm of lumen diameter per total µm of stolon width per second) for three clonal replicates of each of nine colonies for *P. carnea* (open bars) and *H. symbiolongicarpus* (filled bars) at a stage of colony development comparable to stage 3. Colonies of the two species are paired arbitrarily.

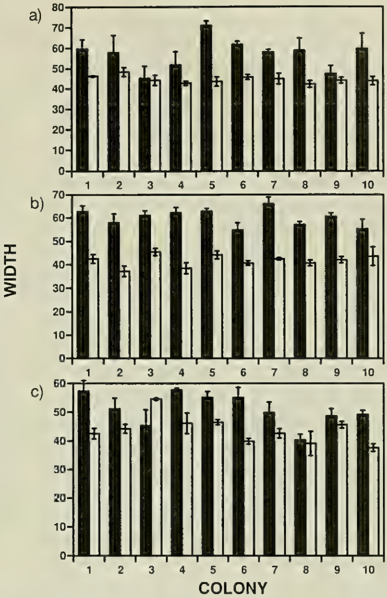

Figure 7. Means and standard errors of periderm-to-periderm stolon widths (µm) for 10 colonies of each of *P. carnea* (open bars) and *H. symbiolongicarpus* (filled bars) at 3 stages of colony development (a = stage 1, b = stage 2, c = stage 3). Colonies of the two species are paired arbitrarily.

It is also worth examining the assumption that the volume of the stolon tip scales in proportion to the measured diameter and not in proportion to its square (see discussion in Methods). If, for instance, it is assumed that the cross-sectional area of the stolon lumen is always circular, then the change in stolon tip volumes would be proportional to the maximum lumen radius squared minus the minimum lumen radius squared. These values can be substituted for amplitude (maximum diameter minus minimum diameter) in the above analysis. If this is done (using a square root transformation to meet the assumptions of parametric statistics), similar results are obtained. In stage 1, *H. symbiolongicarpus* has greater values ($F = 23.06$, $df = 1, 18$, $P < 0.001$). In stage 2, there is no between-species difference ($F = 3.36$, $df = 1, 18$, $P > 0.05$). In stage 3, *P. carnea* has greater values ($F = 20.75$, $df = 1, 18$, $P < 0.001$). Using estimates of volume based on squared measures, without adjusting for stolon width, exaggerates the effects of the larger stolons of *H. symbiolongicarpus,* but does not change the overall conclusions. This is as expected because the measures of differences between linear distances are highly correlated to the measures of differences between squared distances, *e.g.,* for stage 1, $R^2 = 0.88$. Given the observed behavior of the stolon lumen in contraction cycles, however, linear measures are preferred.

Finally, while this study focused on between-species differences, it may be that within-species differences in morphology and life history may also relate to the rate of gastrovascular flow. This is most likely in *H. symbiolongicarpus,* where there is substantial within-species variation. The rate of stolonal expansion and contraction at the time of stolonal mat formation is a significant predictor of both the amount of stolonal mat that forms at this time (Fig. 8a; Spearman's $R_s = -0.66$, $P < 0.04$) and the time at which the colony will subsequently reproduce (Fig. 8b; Spearman's $R_s = -0.7$, $P < 0.03$). The possibility that the timing of reproduction in these colonies can be predicted, even approximately, by 30 minutes of filming at the time of stolonal mat formation is intriguing.

Additional morphological and life history comparisons

Compared to *P. carnea,* the *H. symbiolongicarpus* colonies have a greater mean time from metamorphosis to the appearance of mature gonozooids (mean ± standard error in days; 53.2 ± 1.22 *versus* 68.4 ± 7.09). While this difference is marginally significant (using natural log-transformed values, $F = 4.6$, $df = 1, 18$, $P < 0.05$), the most notable difference is in the range of values (for *P. carnea,* 50–60 days; for *H. symbiolongicarpus,* 50–106 days). Further, compared to *P. carnea,* the basal portions of the *H. symbiolongicarpus* colonies are more optically dense, *i.e.,* they transmit less light (Fig. 9; testing the between-species effect against the colony-within-species effect; $F = 5.78$, $df = 1, 18$, $P < 0.03$). These differences likely relate to the commonly observed dark-colored deposits of the spine-forming material (likely a chitin-protein complex) in *H. symbiolongicarpus.*

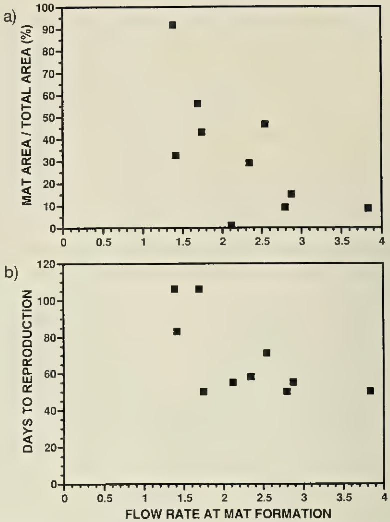

Figure 8. Bivariate scatter plots of the means of rates times 10^3 of lumen contraction and expansion (μm of lumen diameter per total μm of stolon width per second) for 10 colonies of *H. symbiolongicarpus* at stage 2 and (a) the amount of stolonal mat which forms at stage 2, and (b) the total number of days from metamorphosis to reproduction.

Mechanisms relating flow to colony development

The ten "pre-primary" polyps show a rate of expansion and contraction of the stolon lumen significantly different from zero (mean ± standard error of rates times 10^3; 2.11 ± 0.18; note that roughly twice the standard error yields a 95% confidence interval). While this rate is less than that of the *H. symbiolongicarpus* primary polyps (Fig. 4a), these pre-primary polyps were unfed prior to filming (flow rate reaches a maximum shortly after feeding and subsequently declines, see Schierwater *et al.,* 1992). On the other hand, in clonal replicates of field-collected colonies there is no correlation between the rate of expansion and contraction of the stolon lumen and the amount of stolon tip growth if this rate is adjusted for the size of the stolon (*i.e.,* relative amplitudes/periods, Fig. 10a; for *H. symbiolongicarpus,* $R^2 = 0.05$, $F = 2.6$, $df = 1, 52$, $P > 0.11$; for *P. carnea;* $R^2 = 0.0002$, $F = 0.013$, $df = 1, 52$, $P > 0.9$). If this rate is not adjusted for the size of the stolon (*i.e.,* amplitude/period, Fig. 10b), there is a slight but statistically significant correlation for *H. symbiolon-*

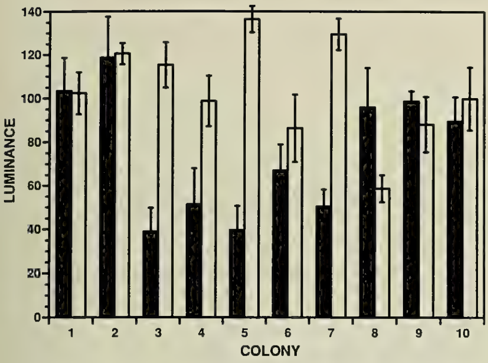

Figure 9. Means and standard errors of the average luminance (on a gray-level scale of 0 to 255) for 5 measured areas of each of 10 colonies of *P. carnea* (open bars) and *H. symbiolongicarpus* (filled bars) at the time of the development of mature gonozooids. Visible light with a luminance of 165 was transmitted through each colony; areas of polyps or empty coverslip were not measured.

gicarpus ($R^2 = 0.09$, $F = 5.28$, df = 1, 52, $P < 0.03$) and no significant correlation for *P. carnea* ($R^2 = 0.01$, $F = 0.71$, df = 1, 52, $P > 0.4$). The significant correlation for *H. symbiolongicarpus* is the result of two outliers (Fig. 10b). If these outliers are removed from the analysis, the correlation disappears ($R^2 = 0.03$, $F = 1.57$, df = 1, 50, $P > 0.2$). There is also no significant between-species difference in tip growth (using the colony-within-species effect as the error term, $F = 1.01$, df = 1, 16, $P > 0.3$).

Discussion

Hydractiniid hydroids show morphological and life history variation characteristic of many colonial taxa. Previous work using clonal replicates of field-collected colonies has shown that compared to *H. symbiolongicarpus*, *P. carnea* colonies grow more quickly and exhibit more irregular shapes (Blackstone and Buss, 1991). In addition, it is now clear that at the time of stolonal mat formation in *H. symbiolongicarpus*, *P. carnea* colonies form more irregular, "runner-like" shapes, have fewer polyps, and have larger areas of empty substratum enclosed within the colony. These differences persist as colonies mature. Further, *P. carnea* colonies initiate the sexual phase of the life cycle sooner than *H. symbiolongicarpus*, and at maturity the basal portions of *P. carnea* colonies are less of an obstruction to transmitted light, probably because of deposits of spine-forming materials in *H. symbiolongicarpus*. Coupled with other obvious life history differences (*e.g.*, the medusa and swimming planula of *P. carnea*), what emerges is the stereotypical "runner" and "sheet" dichotomy (Buss and Blackstone, 1991); compared to *H. symbiolongicarpus*, *P. carnea* colonies grow rapidly, build a flimsy somatic morphology, reproduce early, disperse widely, and colonize readily.

Previous experimental work with *P. carnea* has suggested that morphological and life history differences paralleling those described above can be induced by perturbing the gastrovascular flow to peripheral stolons with treatments of uncouplers of oxidative phosphorylation and with treatments of increased feeding (Blackstone and Buss, 1992, 1993). These experimental data have led to the hypothesis that flow to peripheral stolons in *H. symbiolongicarpus* will decrease relative to *P. carnea* as colony development proceeds and as the former assumes a sheet-like morphology relative to the latter.

In this context, it is of particular interest to establish the time course of gastrovascular flow as colonies develop in *P. carnea* and *H. symbiolongicarpus*. At the time of stolonal mat formation in *H. symbiolongicarpus*, colonies of this species exhibit a lower rate of contraction and expansion of the lumen of peripheral stolons than colonies of *P. carnea*. At the developmental time at which morphological differences become apparent, the predicted differences in flow are also apparent. These differences in

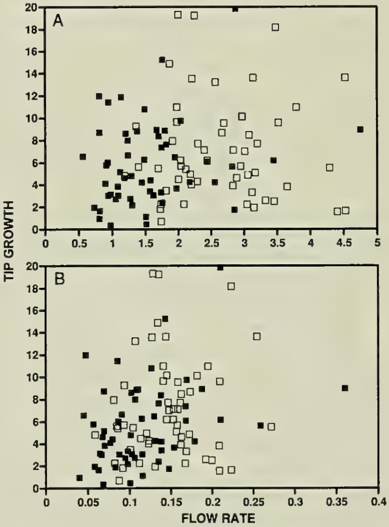

Figure 10. Bivariate scatter plot of tip growth (μm) during a 15-min interval and the mean rate of lumen contraction and expansion for two cycles of each of two stolonal tips for three clonal replicates of nine field-collected colonies of *P. carnea* (open squares) and *H. symbiolongicarpus* (filled squares) at a stage of colony development comparable to stage 3. In (A), rates are adjusted for stolon width (μm of lumen diameter per total μm of stolon width per second) and multiplied by 10^3; in (B) rates are not adjusted for stolon width (μm of lumen diameter per second).

flow rate seem to intensify as colony development proceeds, although flow was not measured at the time of initiating sexual reproduction (in *H. symbiolongicarpus*) or the sexual phase of the life cycle (in *P. carnea*). Energetic considerations suggest that flow might diminish at this time in both species.

The time course of colony development suggests that heterochrony, an evolutionary change in the timing of development, has produced the differences between these species. In particular, both species become more sheet-like as they develop, but *H. symbiolongicarpus* colonies produce a sheet-like morphology well before sexual reproduction is initiated, while *P. carnea* colonies produce a sheet-like morphology only at or near the initiation of the sexual phase of the life cycle. The timing of sheet formation thus seems to have changed in the evolution of these hydractiniid hydroids (*i.e.*, either *H. symbiolongicarpus* has accelerated sheet production or conversely, *P. carnea* has delayed it). A diminishing of gastrovascular flow accompanies the formation of a sheet-like morphology in *H. symbiolongicarpus*. It is not clear if gastrovascular flow diminishes at the time of the initiation of the sexual phase of the life cycle in *P. carnea* (*i.e.*, the time of the formation of a more sheet-like morphology in this species). This would be predicted by the hypothesis of heterochrony.

At the time that stolons first begin to grow in "preprimary" polyps, gastrovascular flow can also be observed. It seems that stolons do not exist without such flow. This result is unsurprizing in view of the stolon's usual reliance on gastrovascular flow for nourishment. Given that stolons require flow for growth, it is tempting to predict that the rate of flow correlates with the rate of stolon growth. The data do not support this hypothesis. There is little or no correlation between the rate of gastrovascular flow and the immediate rate of growth of the stolon tip. At the time tested, there is also no significant difference between *P. carnea* and *H. symbiolongicarpus* in the rate of stolon tip growth. Possibly, a more complex relationship between flow and growth exists.

Nevertheless, it seems that the most plausible mechanism by which gastrovascular flow mediates morphological development is by regulating stolonal branching events and polyp formation. This hypothesis suggests that a high rate of flow suppresses both stolonal branching and polyp formation, while a low rate of flow permits these events. This hypothesis requires a mechanism for gastrovascular flow to signal morphogenetic events, although the nature of this mechanism is not clear.

Acknowledgments

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Ontogeny of Copepod Predation in Juvenile Squid (*Loligo opalescens*)

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Abstract. Copepods are the major prey of juvenile squid, and small species of squid such as *Loligo opalescens* face a great challenge in catching these erratically moving crustaceans. We studied the ontogeny of copepod predation in laboratory-reared animals and found that mastery of copepod capture develops progressively, starting shortly after hatch with strong attacks of a simple type. Modifications of the initial basic attack lead to more specialized strategies that effectively extend the range of capture to both longer and shorter distances. This progression culminates, by approximately 40 days post-hatching, in adult-like prey capture behavior involving tentacle extension and retraction. Squid raised exclusively on easily captured *Artemia* nauplii and introduced to a copepod diet 40 days after hatching displayed only basic attack behavior, characteristic of very young squid. All of these attacks were unsuccessful, and very few of these animals survived the transition. Copepod capture thus appears to be a skill that must be acquired in an experience-dependent manner early in post-hatching life.

Introduction

Prey capture behavior has been studied in adult *Loligo opalescens* (Fields, 1965) and a number of other pelagic squid species including *L. vulgaris* (Neill and Cullen, 1974), *L. pealei* and *L. plei* (Kier, 1982), *Ilex illecebrosus* (Nicol and O'Dor, 1985), *Sepioteuthis sepiodea* (LaRoe, 1971), and *Sepioteuthis lessoniana* (Lee *et al.*, 1994). In each case, prey capture resembles that of the cuttlefish, *Sepia officinialis*, which displays sequential phases of attack behavior: (i) attention and orientation, (ii) positioning and approach, and (iii) seizure and capture (Messenger, 1968). The first two sets of locomotor behavior are carried out by fin movements and jet propulsion; the latter phase is mediated by the tentacles and arms. All phases of the attack, however, are far from simple or stereotyped. They are typically directed at very high speed against organisms such as shrimp and fish that possess well-developed escape responses. Moreover, the exact strategy of attack can be modified depending on the size and speed of the prey (Foyle and O'Dor, 1988), on whether the prey organism is part of a school (Neill and Cullen, 1974), and probably on many other factors (Moynihan and Rodaniche, 1982).

Like the adults, juveniles of *L. opalescens* are also active predators with a high metabolic rate sustained by the consumption of 35%–80% of their body weight per day (Hurley, 1976; Yang *et al.*, 1983, 1986). These laboratory rearing studies have revealed that hatchling squid vigorously hunt and attack copepods, a major component of the marine plankton. Although little is known about the early life history of this species, including its diet (see also Vecchione, 1981), prey in the wild undoubtedly consists of a variety of planktonic species, including copepods. Because copepods move erratically and display extremely quick escape responses triggered by rapid motion of nearby predators (Yen and Fields, 1992), capture of this natural prey must pose a considerable challenge for a newly hatched squid.

Behavior as complex as prey capture in cephalopods is likely to have both instinctive and experience-dependent or activity-driven components (Wells, 1962; Messenger,

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Abbreviations: ML, Mantle length.

1977), but the relative importance of these components has not been deeply investigated in squid. Hurley (1976) studied prey capture behavior in very young *L. opalescens*, observing attacks on *Artemia* nauplii, copepods, and larval fish. She found that the general three-stage pattern described above was already displayed immediately after hatch and that the success rate for *Artemia* capture increased markedly over the first 30 days post-hatching, from near zero to about 50%.

In this paper we study more thoroughly the nature of predatory attacks on copepods by *L. opalescens*, focusing on the ontogeny of this complex behavior during post-hatching development. In particular, we examine the performance modifications that occur in squid as their motor capabilities develop after birth, show how these changes lead to improved success in copepod capture, and examine the importance of experience in the development of predatory behavior.

Materials and Methods

Overall experimental design

This study involved the simultaneous rearing of two experimental groups of hatchling squid. Wild plankton, primarily composed of copepods, was fed to one group from hatching onward (Group 1), whereas only *Artemia* were fed to the second group of squid from hatch until day 40 (Group 2). On day 40, the feeding of *Artemia* was ended, and Group 2 squid were fed on wild plankton thereafter.

This experimental design allowed us to observe developing prey capture techniques in squid exposed to copepods from birth compared with those found in the squid provided only with slow-moving *Artemia*. Study of attacks on copepods by squid raised only on *Artemia* provides additional insight into the experience-dependent aspects of the ontogeny of prey capture.

Results described here are based primarily on squid reared during April–June 1994. Data pertaining to copepod predation by newly hatched squid (days 1–4 post-hatching) were obtained in September 1994. A previous experimental rearing during April–June 1993 yielded results consistent with the second run.

Culture

Fertilized eggs of the squid *Loligo opalescens* were obtained from adult, wild-collected animals that were allowed to spawn in circular tanks (2.5 × 1 m) supplied with flow-through ambient seawater (12–15°C) at Hopkins Marine Station. For every experimental run described above, about five egg cases were transferred into each of two identical 320-liter tanks at the Monterey Bay Aquarium: the hatchling squid in each tank constituted an experimental group. These black cylindrical tanks, 1 m in diameter, possess gently sloping conical bottoms and were exposed to natural daylight and, between 6 a.m. and 6 p.m. daily, to overhead fluorescent illumination. Except for periods of maintenance and feeding, the tanks were kept partially covered with a black plastic sheet. Organic debris was skimmed from the surface and siphoned from the bottom of each tank daily, and any squid mortalities were counted at this time.

Flow-through water was provided to the rearing tanks from a temperature-controlled reservoir (300 liters volume) fed with natural seawater passed through a 10-μm filter at a rate of 1–1.5 l/min. Egg cases were held at 14–15°C until hatching, after which the temperature was increased by 0.6°C. per day to 17–18°C. Once hatching commenced, the eggs were removed after several days, and day-one post-hatching was assigned to the day of most significant hatching activity.

Feeding

Squid hatchlings were fed once daily, either with *Artemia* nauplii enriched with algae and Super Selco, a nutrient medium rich in lipids, fatty acids, and vitamins (Artemia Systems N.V., Belgium), or with live marine plankton. Plankton was obtained by conventional surface tows in Monterey Bay and consisted primarily of small *Acartia* sp. copepods, although larger calanoid copepods, chaetognaths, and assorted crustacean larvae were also present to a variable extent. One group of hatchlings (Group 1) was fed plankton as regularly as possible and *Artemia* when plankton was not available. The second group of hatchlings (Group 2) was fed only *Artemia* until day 40, when their diet was switched to plankton.

Filming

Feeding behavior was recorded during sessions lasting approximately 30 min with a Canon A1 Digital Hi8 camcorder fitted with a #1 close-up lens, mounted on a tripod, and operating at 30 frames per second with a shutter speed of 1/60 second. A 500-watt halogen floodlight mounted 1 m overhead provided supplemental lighting; intensity was adjusted with a transformer. The camcorder was elevated on the tripod and turned downward so that filming occurred at an angle almost perpendicular to the water surface. Well-illuminated squid displaying attack behavior were visually identified and selectively filmed with the camera fixed in position until the squid either successfully caught the prey item, stopped attacking, or moved out of the field of view. Slow vertical changes of the squid and prey item were followed to a maximum depth of 30–40 cm by manually changing the focus and zoom of the camera.

Frame-by-frame data analysis was undertaken using a Sony 9700 Hi 8 editing deck and a Trinitron monitor.

Attack distance, defined as the straight-line distance between the tip of the squids' arms and the copepod on the frame preceding the actual attack ("d" in Fig. 2), was measured off the monitor using a dial caliper precise to 0.00254 cm and normalized to the mantle length (ML) of the squid. Attack speed was computed as the greatest distance traveled by the squid between two consecutive frames during the attack lunge and is expressed in ML/frame. Attacks in which the squid was obviously moving vertically to a significant extent were not analyzed.

Results

Mortality of early post-hatching squid

Figure 1 indicates the feeding regimen for each tank (1994 run) and presents the daily population (back-calculated at the end of the experiment) and mortality counts. Mortality peaks occur over days 1–12 and 25–35 in both groups, and similar results have been reported in other studies (Hurley, 1976; Yang *et al.,* 1986; Hanlon and Hixon, 1983). The large number of deaths in Group 2 (dotted line and open triangles) starting on day 40 is discussed below. Similar results were obtained with the same type of feeding manipulations in the 1993 rearing.

Development of attack strategy: the basic attack sequence

Shortly after hatching, the basic attack sequence illustrated in Figure 2A emerges. Initially, a squid exhibits an attention or arousal type response (Lynn, 1966) manifested as an active orientation towards the potential prey item, accompanied by a pointing of the arms in a cone directly at the target. This posture is maintained through rhythmic jetting and fin beating as the prey item drifts, and the squid thus remains in a fixed position relative to the prey. As the actual attack commences, the squid opens its arms over a period ranging from one to four video frames and then swiftly jets forward at the prey item, typically also within four frames. An attack ends successfully when the squid snaps its arms shut and captures the prey, or unsuccessfully, when the prey responds to the attack with a quick escape response and eludes the squid.

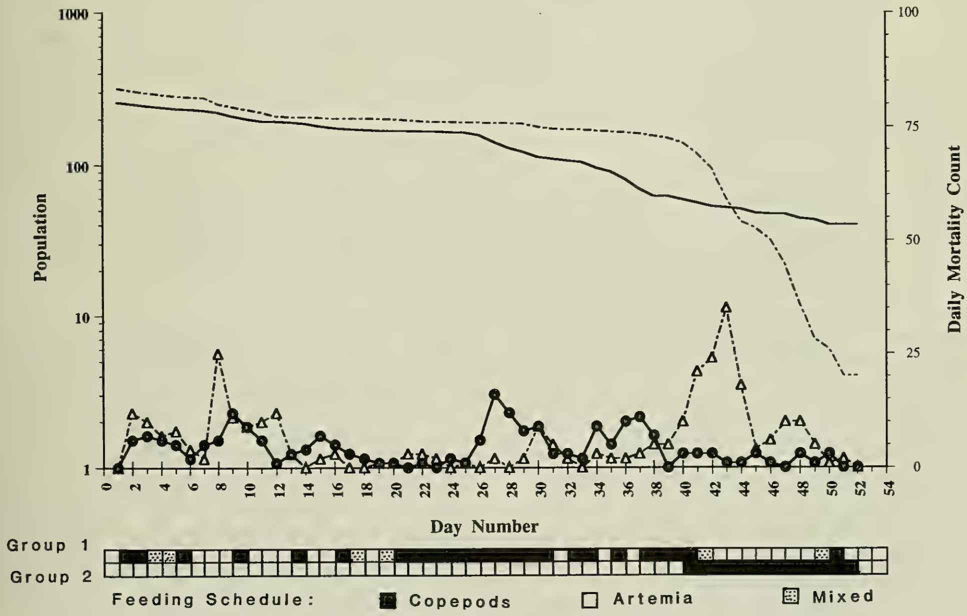

Figure 1. Survivorship of squid from experimental Groups 1 and 2. Surviving population number is plotted on the left-hand vertical axis for copepod-fed, Group 1 (solid curve) and *Artemia*-fed, Group 2 (dashed curve) squid. The exact feeding regimens are encoded in the two bars directly beneath the horizontal axis. Daily mortality counts are also plotted (right-hand vertical axis) for Group 1 (filled circles) and Group 2 (open triangles). Note the dramatic increase in Group 2 mortality after the switch to a copepod diet on day 40.

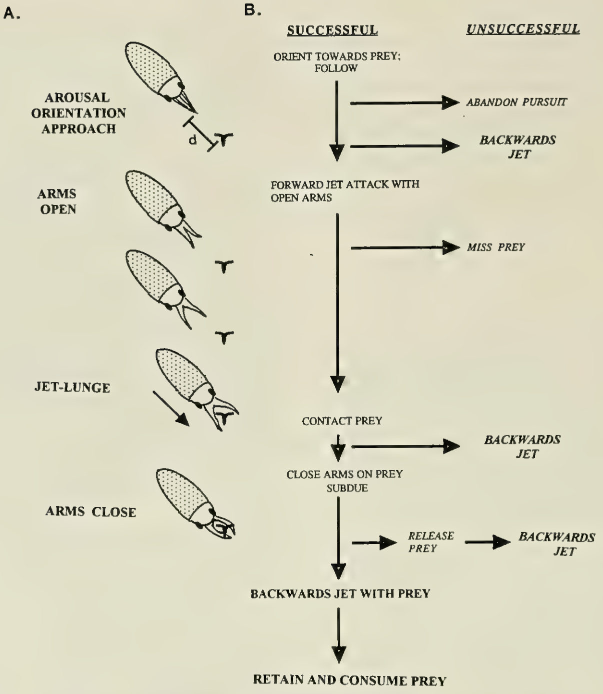
A.
AROUSAL
ORIENTATION
APPROACH
d
ARMS
OPEN
JET-LUNGE
ARMS CLOSE
B.
SUCCESSFUL
UNSUCCESSFUL
ORIENT TOWARDS PREY; FOLLOW
ABANDON PURSUIT
BACKWARDS JET
FORWARD JET ATTACK WITH OPEN ARMS
MISS PREY
CONTACT PREY
BACKWARDS JET
CLOSE ARMS ON PREY SUBDUE
RELEASE PREY
BACKWARDS JET
BACKWARDS JET WITH PREY
RETAIN AND CONSUME PREY

Figure 2. The basic attack sequence. (A) This schematic outlines the first attack behavior to appear within a few days of hatching. It is used in attacks on both copepods and *Artemia* nauplii. Attack distance is indicated by "d" (see Methods). Basic attacks are often driven by very strong jetting behavior. (B) The behavioral sequence depicted in panel A is represented in flow-chart form. Vertical arrows indicate the progression to successful prey capture. Horizontal arrows represent unsuccessful outcomes, a number of which result from improperly timed backwards jets (see text for details).

This attack sequence is the most general strategy of prey capture displayed by newly hatched squid and is the first to appear, regardless of prey type provided. During the first few days of exposure to prey, most attacks are unsuccessful, but the basic attack becomes refined and more successful during repeated attempts. Analysis of the earliest feeding attacks provides a more detailed view outlined in Figure 2B. This flow-chart defines the steps necessary for successful prey capture and identifies commonly observed alternative behaviors that result in failed attacks on both *Artemia* nauplii and copepods.

During the orientation and following phases, the squid may simply abandon the attack, swimming or drifting off in another direction. Squid that have advanced in the sequence to the point of opening the arms commonly abort the attack with a rapid backwards jet that probably represents an escape response in reaction to sudden movement of the prey. This behavior was commonly seen in squid attacking fast-moving copepods, whereas animals feeding on *Artemia* more often abandoned the pursuit. Once an attack is launched, the squid can err by missing the target due to a poorly aimed jet, a jet too weak to cover the attack distance, or one too strong that results in overshooting the prey. Even if the attack jet is well executed, the squid must successfully grasp the prey in its arms in order to make a capture, and this presents a final critical point. Squid commonly contact the prey and generate a vigorous backwards jet before successfully closing the arms. Occasionally they also release the prey after an apparently successful enclosure and follow this with a strong backwards jet. A successful attack in which the prey is subdued is also invariably followed by a quick backwards jet before the squid begins consuming the prey during normal slow swimming.

Unsuccessful attacks as described above are commonly observed soon after hatching when squid are first exposed to prey of any type, or in *Artemia*-reared squid exposed to fast-moving copepods for the first time (see below). In addition to outlining the temporal sequence of attack behavior, the flow chart in Figure 2B also approximates an ontogenic sequence by which the basic attack is perfected after hatching.

Refinement in positioning for attack: circling

Attacks on both copepods and *Artemia* nauplii by squid of all ages studied were attempted from all directions, but the preferred direction of attack changed over time in the Group 1 squid feeding on copepods. During the earliest feeding trials (days 2–4) most attacks were attempted from the posterior end of the copepod (Fig. 3A1). Within about one week, however, the frequency of head-on attacks (towards the anterior of the copepod) began to increase, and head-on attacks became quite prominent, acounting for about one third of all attempts after day 26 (Figs. 3A2, 3). Successful attacks (open circles in Fig. 3A) appear to be more common with the head-on approach in these older squid.

Development of the preference for head-on attacks was not seen in the Group 2, *Artemia*-fed squid and emerged in Group 1 animals as a variation in the basic attack behavior that we have labeled "circling" (Fig. 3B). In this case, a squid pursuing a copepod would move angularly, rotating around the copepod, apparently trying to attain a head-on position (1). During this process, the copepod often reacted to the squid's approach by jumping forward at an angle, propelling it out of the squid's line of approach (2). In response, the squid would shift laterally and again approach the copepod, repeating the rotation behavior in another attempt to position itself in front of the copepod (3). This sequence of action and reaction often repeated itself many times, with the pair of animals circling in an expanding spiral (4–9). This interactive behavior eventually culminated in an attack by the squid, but unsuccessful attacks were often immediately followed by another cycle of circling.

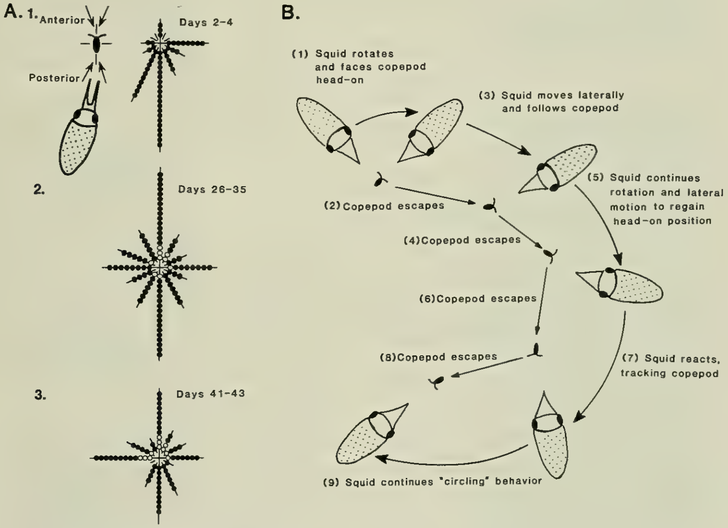
A. 1.
Anterior
Posterior
Days 2–4
2.
Days 26–35
3.
Days 41–43
B.
(1) Squid rotates and faces copepod head-on
(2) Copepod escapes
(3) Squid moves laterally and follows copepod
(4) Copepod escapes
(5) Squid continues rotation and lateral motion to regain head-on position
(6) Copepod escapes
(7) Squid reacts, tracking copepod
(8) Copepod escapes
(9) Squid continues "circling" behavior

Figure 3. Positioning for an attack and "circling" behavior. (A) The direction from which attacks on copepods are launched changes over several weeks post-hatching, and head-on attacks become common. Direction of attack by Group 1 squid was estimated (to the nearest "hour" of a 12-hour clock face centered on the prey) from those video segments in which orientation of the copepod could be identified. Each filled circle represents one unsuccessful attack; successful attacks are indicated by open circles. The orientation of each data clock matches that of the copepod in the inset. 1: Days 2–4 post-hatching; 2: Days 26–35; 3: Days 41–43. (B) Before lunging at the prey item, developing squid often maneuvered in a rotating manner as indicated in order to attain position in front of the copepod prey (1). This positioning of the squid commonly elicited an escape response by the copepod (2), followed by a lateral adjustment of position by the squid (3) rotation previously described. This cycle was often repeated many times (4–9) before a definitive attack was launched by the squid, and the trajectories of the two animals formed a circular or spiral path.

Development of more specialized attack strategies

Once circling was successfully incorporated into the basic attack sequence, at least two specialized forms of attack behavior emerged. As in the case of circling, these behaviors were regularly observed with copepod-fed squid only.

In squid older than 29 days, a novel use of the arms to catch copepods was common (10 of 38 successful attacks analyzed; see also below). Sequential video frames of an "arm-intercept" attack are shown in Figure 4 and schematic representations are illustrated in Figure 4B. After positioning itself in front of a copepod (1) and opening its arms extremely wide (in comparison to a basic attack) (2), a squid would make a quick attack jet towards the copepod (3). This, in turn, triggered a forward-directed escape response by the copepod that often led to a collision with the squid's outstretched arms. A fast reaction by the

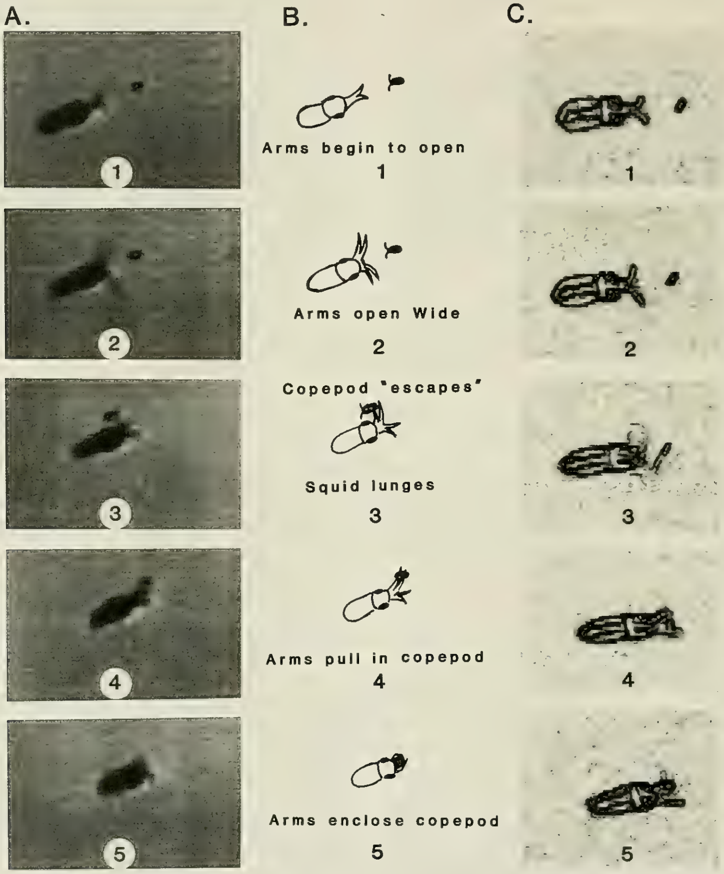

Figure 4. "Arm-intercept" copepod capture behavior in older squid. This type of capture, appearing only after day 29, involved interception of an escaping copepod and was launched from comparatively long distances (usually > 0.5 ML) using only modest jetting behavior. (A) Sequential video frames of an arm-intercept were sampled from taped data using an LC-3 frame-grabber (Scion Corp.) and NIH Image V1.57 software (NIH, Bethesda, MD). (B) Schematic equivalents of the raw data in (A). (C) Sequential video frames of another example of an arm-intercept were sampled as described above and modified with one pass of "Find Edge," an edge-sharpening algorithm in NIH Image.

squid to pull the prey in (4) and enclose it in the other arms (5) resulted in capture. Another example of an arm-intercept is given by the sequential video frames in Figure 4C. The trajectory of the escaping copepod is visible in frame (3).

Arm-intercepts are thus distinguished from basic attacks by (i) the degree of separation of the squid's arms prior to the attack jet and (ii) onset of the copepod's escape response before contact is made. Successful arm-intercepts were first observed on day 29 and were often launched from relatively long distances (See below). Unsuccessful arm-intercepts were difficult to identify unambiguously, and the earliest developmental appearance of this attack type is therefore unknown.

Oldest squid in the present study perfected an adult-like feeding mode, the "tentacular strike." This behavior is illustrated in the sequential video frames in Figure 5A and their schematic representation in Figure 5B. In this case, the squid would first smoothly maneuver close to the copepod (1), open its arms and slightly extend the tentacles while separating the tips (2). Following this, very rapid extension of the tentacles within 1–2 frames produced a forceful strike upon the copepod, enabling its seizure (3–4). Tentacle retraction then served to pull the copepod into the open arms (5–8). During such an attack, forward movement was almost entirely due to tentacle extension; jet-driven body motion was very slight. Tentacular strike attempts were first observed on day 32, but only one successful capture using this strategy was observed before day 40.

Attack distances and speeds change during development

Many attack sequences directed against copepods were filmed during the 1994 study to determine attack distance and maximum attack speed. A rough correlation between attack speed and distance was found for both successful attacks (diamonds in Figs. 6A–D) and unsuccessful attempts (crosses) at all times in this study, and the strength of the correlation appears to increase with age. Attacks are very seldom successful when launched from distances greater than 1 mantle length (ML), regardless of speed or developmental stage, and such long-distance attempts become progressively less common during days 1–42.

These data are expressed in terms of ML, rather than absolute distance, and the similar slopes in Figs. 6A–D imply that absolute attack speed increases in proportion to ML, which is 2.1–2.2 mm at hatch and doubles over the first 40 days post-hatching in this species raised under similar conditions (Yang *et al.,* 1983, 1986; Hanlon *et al.,* 1987). Ten randomly selected animals from each of Groups 1 and 2 had mean (±1 S.D.) mantle lengths of 3.9 ± 0.3 mm and 4.1 ± 0.7 mm, respectively, on day 31. Absolute attack distance, on the other hand, does not appear to increase as squid grow larger, and the minimum attack distance actually decreases, as evidenced by the lack of points below 0.3 ML in Figure 6A.

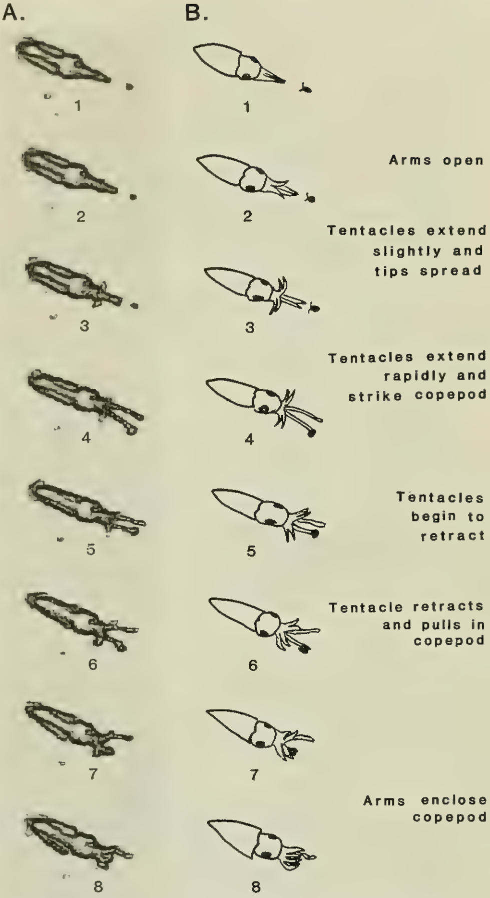
A.
B.
1
1
Arms open
2
2
Tentacles extend slightly and tips spread
3
3
Tentacles extend rapidly and strike copepod
4
4
Tentacles begin to retract
5
5
Tentacle retracts and pulls in copepod
6
6
7
7
Arms enclose copepod
8
8

Figure 5. "Tentacular strike" capture behavior in the oldest squid studied. This style of prey capture is essentially adult-like; the first successful attempts were observed on day 41 post-hatching. These attacks were launched exclusively from short distances (<0.4 ML) and involved minimal jetting behavior. (A) Sequential video frames show a successful tentacular strike. Sampling is described in legend to Figure 4C. (B) Schematic representation of the behavioral sequence in (A).

Decreasing attack distance and a concomitant increase in success during days 1–42 is also seen in an analysis of the type of successful attacks used at various stages. Figure 7 illustrates histograms of successful copepod captures

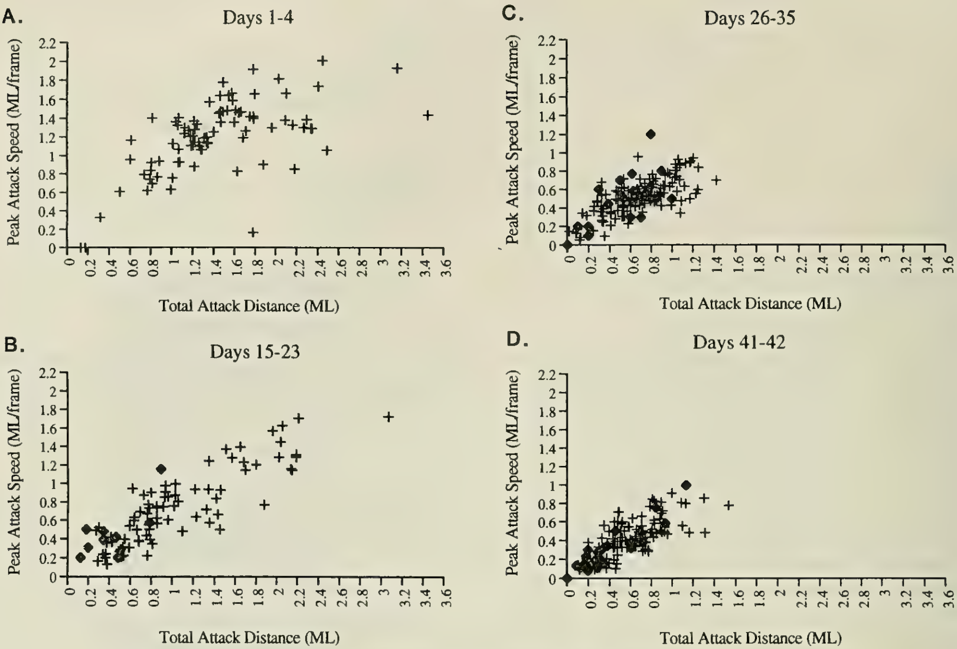

Figure 6. Peak attack speed and attack distance tend to grow smaller as squid mature. Peak attack speed *versus* attack distance (as defined in Methods and Fig. 2) are plotted for plankton-fed Group 1 squid grouped into four categories as follows: (A) Days 1–4; (B) Days 15–23; (C) Days 26–35, (D) Days 41–42. Successful attacks are indicated by diamonds; unsuccessful attempts are denoted by crosses. The progressive loss of long distance attacks is obvious, and there is only one successful attack from beyond 1 ML.

grouped by attack distance for three age classes. During days 15–23 (Fig. 7A), squid employ only basic attacks, and captures occur over a wide range of distances relative to ML. In the intermediate group (days 26–35; Fig. 7B), basic attacks are still regularly employed (12 of 18 captures), but arm-intercept attacks appear during this period and lead to an improved capture rate from long distances. At least 4 of 10 successful attacks above 0.5 ML employed this technique. In the oldest squid (days 41–42; Fig. 7C), basic captures persist, arm-intercepts remain effective at longer ranges, and the marked improvement in short-distance attack reflects successful tentacular strikes (6 of 10 below 0.4 ML).

Figure 8 illustrates complementary data for unsuccessful attacks by squid of similar age classes (Figs. 8B–D) and for newly hatched squid (Fig. 8A). Distributions for unsuccessful attacks superficially resemble those for captures (Fig. 7) but are clearly skewed towards longer distances. This is most obvious in day 15–21 squid (Fig. 8B *versus* Fig. 7A). Longer distance attacks thus have a higher probability of failure, particularly with the basic strategy, and unsuccessful attacks of this type are extremely common in very young squid (days 1–4; Fig. 8A). During this early period attacks from below 1.0 ML are clearly the exception, and no successful captures were observed.

Failure of copepod capture by squid raised on Artemia

Results described in conjunction with Figures 6–8 were obtained on Group 1 squid, which had been fed copepods from birth, and mortality in this group was low throughout the study. Group 2 squid, reared from birth on only enriched *Artemia* nauplii, also showed low mortality up to day 40. On day 40, however, these squid were subjected to a switch in prey type from *Artemia* to copepod-rich, fresh plankton. Immediately following this manipulation, the mortality rate accelerated dramatically. Of the 142 squid present on day 40, only four survived to day 52.

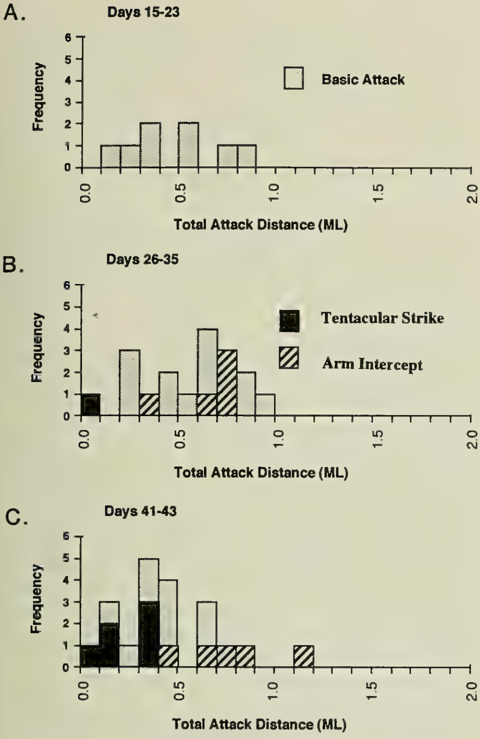

Figure 7. Successful captures launched from short distance become more common as squid mature. Histograms for the number of successful copepod attacks by Group 1 squid are grouped according to attack distance for the age classes defined in the legend of Figure 6: (A) Days 15–23; (B) Days 26–35; (C) Days 41–42. Basic attacks are encoded by dotted bars, arm intercepts by cross-hatched bars, and tentacular grabs by solid bars.

In this experiment, Group 1 and 2 squid were reared simultaneously in adjacent tanks fed by a common source of seawater and were treated identically except for the feeding paradigm. Moreover, this experiment was a successful repetition of one carried out one year earlier. For these reasons, we postulate that the mortality increase in

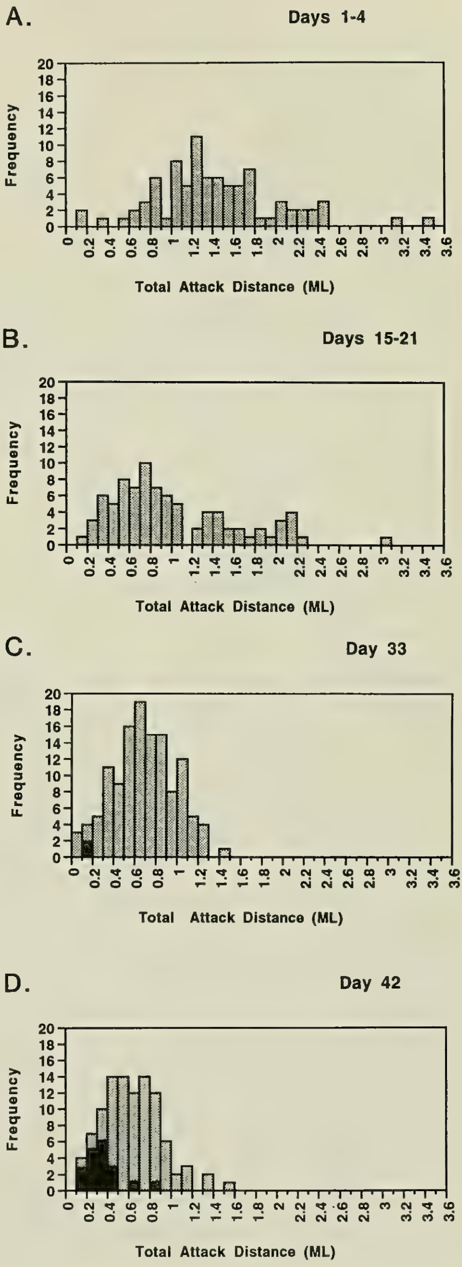

Figure 8. Analysis of unsuccessful attacks. Data for unsuccessful attacks are presented in a format analogous to that of Figure 6 for each age class of Group 1 squid: (A) Days 1–4; (B) Days 15–21; (C) Day 33; (D) Day 42. The coding of histogram bars for attack type is the same as that in Figure 7. Unsuccessful long distance attempts decrease as squid mature, while short-distance attempts increase in frequency.

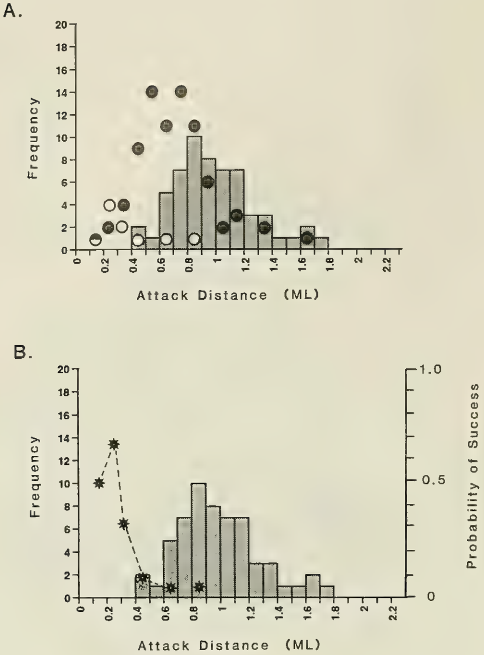

Figure 9. Unsuccessful attacks on copepods after day 40 by *Artemia*-fed, Group 2 squid. (A) Data for Group 2 attacks on days 40–43 following the switch from *Artemia* to copepod prey on day 40 are plotted as the histogram bars. Unsuccessful basic attacks of Group 1 squid from Figure 8D are replotted here as filled circles. Successful basic attacks of Group 1 (from Fig. 7B) are indicated by open circles. The nearly complete lack of Group 2 attacks from short-distance (<0.4 ML) contrasts markedly with the Group 1 data and more closely resembles the pattern for very young Group 1 (Figs. 8A). (B) The histogram for unsuccessful Group 2 attacks on copepods is replotted from panel A along with the probability of success with a basic attack by the Group 1 squid (calculated from data in panel A). Group 2 squid failed to exploit the attack distance range from which a reasonable chance of success exists.

Group 2 beginning on day 40 is a direct consequence of the change in prey.

Behavioral observations of feeding attempts by the Group 2 squid after the switch to copepods support this association. Group 2 squid clearly recognized copepods as potential prey and made many vigorous attacks, often repeatedly attacking the same copepod. Observations on days 41–43 revealed that these attacks were launched exclusively from distances greater than 0.4 ML (Fig. 9A) at speeds appropriate for this range—*i.e.*, distance and speed were correlated (data not illus.)—but no successful attacks were observed.

Consistent failure by these squid clearly resulted from attack sequences directed against copepods that differed in several important ways from those of copepod-reared Group 1 squid of identical age. First, Group 2 squid displayed only stabbing, basic attacks and none of the more specialized attack types using arms and tentacles. Second, Group 2 squid appeared to be unable to approach a copepod closely and did not carefully position themselves before attacking through circling behavior. Instead of stimulating circling behavior, an escape response of the copepod triggered by an approaching squid typically produced an escape response by the squid and an aborted attack. Finally, attacks were predominately launched from long distances.

Comparison of Group 2 data from days 40–43 (Fig. 9A, bars) with those for unsuccessful basic attacks by Group 1 squid of the same age (filled circles) reveals a distribution of unsuccessful attacks in Group 2 squid that is skewed towards long distances with no attempts below 0.4 ML. By day 42, short-distance attacks had become the most successful range for copepod-fed Group 1 squid, and the distribution of successful basic attacks for Group 1 (days 40–42) is given by the open circles. Figure 9B compares the distribution of unsuccessful Group 2 attacks (data of Fig. 9A) with the probability of success for a basic attack derived from the Group 1 data in Figure 9A. Although probability of a successful basic capture is fairly high at short distances, the chance of success falls to near zero beyond 0.4 ML. Thus, Group 2 squid attempted no attacks whatsoever from distances where a reasonable chance of successful copepod capture exists.

Discussion

Results of this study indicate that copepod capture by hatchling squid develops through a progression of attack strategies, beginning with a rudimentary form of the basic attack sequence and ending with an assortment of more elaborate versions as well as tentacular strike attacks similar to those used by adults. In general, the trend is loss of powerful, long-distance attacks that appear never to be successful, and the concomitant increase in short distance attempts that do not require a powerful attack jet. In the limit, the tentacular strike has essentially no forward-directed attack jet. This trend probably increases success of prey capture by reducing the likelihood of triggering an escape response by the copepod and may also help to minimize energy expenditure in making a successful capture.

A correlation between attack distance and speed is evident at all stages (Fig. 6). This strongly suggests that a squid can accurately gauge the distance to its intended prey and adjust attack speed accordingly, so as not to undershoot or overshoot the target. The apparent tightening of this correlation during development, especially over the first 20 days, suggests that a squid's ability to coordinate sensory inputs and motor outputs improves considerably during this period.

Quantitative data on attack speeds and distances in this paper have been measured in terms of ML. In other rearing studies with *L. opalescens* under similar conditions (Yang *et al.,* 1983, 1986; Hanlon *et al.,* 1987), this parameter increased ~40% between days 4–20 post-hatching and an additional 25% between days 20–30 and between days 30–40. For several reason, this growth cannot account for a number of conclusions discussed here.

First, the lack of attacks from <1 ML in day 1–4 squid (Fig. 8A) is still evident if the histograms for unsuccessful attacks by day 15–21 squid (Fig. 8B) are adjusted for the increase in ML by scaling by a factor of 1.4 (scaled data not illustrated). Younger squid simply attack less frequently from short distances, even on an absolute scale.

Second, the increase in successful short-distance attacks (< 0.4 ML) between days 26–35 (Fig. 7B) and 41–43 (Fig. 7C) is accompanied by only a minor increase in body size. Successful incorporation of tentacular strikes explains this difference.

Third, nearly complete absence of copepod-directed attacks from <0.6 ML in the Group 2 squid on days 41–43 contrasts markedly with the distribution of unsuccessful basic attacks made by Group 1 squid of the same age and similar body size (Fig. 9A). Thus, squid that lack experience with copepods, like newly hatched squid, do not launch attacks from short distances even though they are of an age that normally successfully exploits this distance range.

Perfection of attack skills and value of experience

Although the fundamental features of the basic attack appear very shortly after hatch (sometimes on the same day) and may in large part be innate, improvements in performance and development of new strategies appear to be highly activity-driven and experience-dependent. Each progressive step in the ontogeny of copepod capture is refined over the course of many unsuccessful trials, and positive reinforcement of the occasional successful capture must be a powerful driving influence. In turn, refinement of one step leads to a new step and another level of complexity, ultimately resulting in a complex suite of behaviors that together constitute prey capture.

These ideas are well illustrated by the development of circling behavior, leading from attacks launched primarily from behind a copepod to those initiated from a head-on approach that have a much higher probability of intercepting an escaping copepod. In turn, the final attack style also progresses from vigorous, properly timed basic attacks to arm-intercepts and finally to tentacular strikes.

The increased chance of copepod interception with a head-on approach clearly takes advantage of the copepod's stereotyped forward-directed escape response (Yen and Fields, 1992), and adjustment by the squid to unsuccessful attacks from other directions must be a strong driving force in modifying behavior. This is supported by the failure of 40-day-old squid that had been reared entirely on *Artemia* (Group 2) to execute circling behavior and short-distance attacks when fed copepods. Although these squid were highly successful in capturing slowly moving *Artemia,* this was accomplished entirely by basic attacks of the type displayed for only a few days after hatching in Group 1 animals. More specialized attacks such as arm intercepts or tentacular grabs were never observed with either *Artemia* or copepods. Apparently Group 2 remained behaviorally stunted at a performance level characteristic of very young squid that were similarly inexperienced with copepod capture.

Execution and suppression of backwards jets in prey capture

Backwards jetting that results in a failed attack is a common feature during the early stages of perfecting the basic attack sequence (Fig. 2B). Suppression of these escape-like responses is essential to successful execution of an attack. Squid that are in this phase of development often launch a strong, well-aimed, properly timed attack jet and hit the prey head-on, but then execute a fast backwards jet before the arms can engulf the prey.

Backwards jets under these circumstances may be genuine escape responses to painful stimulation of the squid's sensitive mouthparts and arms by the hard exoskeleton and spines of the prey: nevertheless, they prevent successful capture and must be suppressed. Control over the timing of this suppression would appear to be crucial. Whereas backwards jetting *before* successfully engulfing the prey with the arms guarantees failure, a strong backwards jet executed *after* subduing the prey is the normal culmination of a successful attack (Fig. 2B). In the latter case, the backwards jet may remove the predator and its captured prey from the site of a struggle that could potentially attract the attention of nearby larger predators on squid. This feature is also seen in the feeding behavior of adult *L. opalescens* and both hatchling and juvenile *Sepioteuthis lessoniana* (unpub. obs.) as well as *Nautilus pompilius* (O'Dor *et al.,* 1990).

Incorporation of fast jetting behavior, both forward and backward, into prey capture is thus commonplace, and this motor output shares much in common with escape behavior. Presumably the same motor elements are used in both behaviors, and it would benefit either behavior to employ concerted use of small axon and giant axon motor systems (Otis and Gilly, 1990), because the two systems acting together boost performance. Tight control over excitation of the giant axon pathway is crucial to this ability, and similar mechanisms that underlie this control may also be operative in suppressing inappropriately timed backwards jets during feeding attempts in developing squid. Concerted small and giant axon-driven escape responses normally develop shortly after birth (Gilly *et al.*, 1991), during the same period when the basic attack is being perfected and backwards jetting is becoming increasingly suppressed. Control of the giant axon system is undoubtedly central to both escape and prey capture behavior.

Timing of experience during development and its impact

In this study, more than 90% of the *Artemia*-reared Group 2 squid died during the 10 days after the *Artemia*-to-copepod switch, undoubtedly due to starvation and exhaustion brought on by failure to capture copepods with a strategy that had been successful with *Artemia*. This 10-day period is similar to the amount of time over which newly hatched squid normally develop circling behavior and other attack skills that guarantee survival beyond this critical early period following exhaustion of their internal yolk reserves. Thus, the positive impact of both failed and successful copepod attacks on ontogeny of prey capture appears to be far stronger early in life than at day 40.

To what extent significant benefits of experience in copepod capture can be derived by naive squid after a period of 40 days remains an important question. In the present study we were unable for logistical reasons to follow the four surviving Group 2 squid beyond day 52 when the experiment was terminated, but these few individuals may have been unusually plastic or could have attained the equivalent of copepod attack experience in some other manner. Similarly, it remains to be determined if mastering capture of fish and other challenging prey types involves a progression through an entirely different set of attack strategies. If so, it will be important to identify the time when this can successfully occur, and to question whether lack of early experience also leads to impaired performance later in life.

In studies of postnatal development of vertebrate sensory and motor systems, the "critical period" concept is well established (Kandel, 1985), for example in mammalian visual discrimination (LeVay *et al.*, 1980) and in birdsong learning (reviewed by Margoliash, 1987). It would not be surprising if such a phenomenon also existed in invertebrates with neural capabilities as complex as those of squid and other cephalopods, and perhaps in far "simpler" forms as well. In many cases it may be difficult to associate particular behaviors with specific experiences, but this may reflect difficulties with ethological approaches more than limitations of invertebrate nervous systems.

Results described in this paper indicate that ontogeny of copepod capture by developing squid is highly contingent on experience early in life, and that this experience may be of limited value after an early formative period. Indeed, "Such sensitivity of the nervous system to the effects of experience may represent the fundamental mechanism by which the organism adapts to its environment during the period of growth and development" (Wiesel, 1982).

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The Relationship Between Predator Activity State and Sensitivity to Prey Odor

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Abstract. Predators searching for prey commonly alternate periods of endogenous locomotory activity with rest. We examined the effects of activity state on behavioral responses to prey odor by predatory blue crabs (*Callinectes sapidus*) and spiny lobsters (*Panulirus interruptus*). All animals to be tested were placed individually in large seawater tanks (1.5-m diameter) outdoors, where they were assayed for their responses. Initial experiments were conducted at night, the period of greatest normal endogenous activity, in either moonlight or dim, far-red illumination. Prey odor was presented to crabs and lobsters as they either spontaneously walked or rested (for 15–30 min) between locomotory bouts. Only walking animals significantly responded; they either turned towards the site of odor delivery or probed the substratum with chelae and legs. An additional experiment was conducted by presenting a purified prey attractant (ATP) to spiny lobsters. Remarkably, ATP was 498 times more potent in evoking response from walking animals than from resting ones. Neither the duration (15–30 min compared with 4–8 h) nor the timing (night compared with day) of the rest period influenced the sensitivity of the behavioral response. On the basis of our current results, chemical stimuli appear principally to *modulate* searching by predators already aroused rather than to *initiate* foraging or feeding from the quiescent state. Significantly, whenever experiments restrict the activity of freely ranging animals, determinations of chemosensory-stimulated behavior may substantially overestimate the concentrations needed to evoke responses.

Introduction

Chemical cues are important factors mediating predator–prey interactions. The behavioral responses of marine predators to feeding attractants have frequently been reported (see reviews by Croll, 1983; Carr, 1988; Zimmer-Faust, 1989). Because chemical analysis of dilute attractant material in seawater is difficult, attractants released by live prey are not yet fully identified. For this reason, investigators have emphasized studies on predator reactions to either chemically undefined prey odors (Luczkovich, 1988; Vadas *et al.,* 1994) or identified attractants residing at high concentrations in homogenates and extracts of prey tissues (Mackie *et al.,* 1980; Carr and Derby, 1986; Thorington and Hessinger, 1988; but see Carter and Steele, 1982; Rittschof *et al.,* 1984).

Investigators studying chemoreceptive behavior of marine organisms are faced with a challenge: at macroscopic scales, the chemical stimulus environment in flowing water is very difficult to control. Turbulent mixing dilutes chemical stimulus concentration and creates patchiness in stimulus distribution (see review by Okubo, 1980). The magnitude of turbulence increases proportionally with both flow speed and size (diameter or width) of bioassay chambers. To reduce turbulence, investigators have often constructed experimental devices (such as Y-tube olfactometers) to deliver water flow at slow speeds through narrow channels (*e.g.,* Mackie and Shelton, 1972; Chase and Wells, 1986; Benfield and Aldrich, 1991). Although these devices increase investigator control over chemical-stimulus environments, they can restrict the mobility of freely ranging animals.

Laboratory studies of responses to feeding attractants, especially by marine invertebrates, have commonly depended on the initial quiescence of the test animals (Pear-

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son and Olla, 1977; Mann *et al.,* 1984; McClintock *et al.,* 1984; Zimmer-Faust *et al.,* 1984; Fine-Levy *et al.,* 1988). This procedure is meant to simplify the identification of response, but the results may be misleading when the primary effect of a chemical stimulus is to modify the locomotory behavior of animals already aroused, rather than to initiate foraging or feeding from the quiescent state. For example, decapod crustaceans commonly flick the lateral flagella of their antennules upon detecting a chemical stimulus (Price and Ache, 1977; Schmitt and Ache, 1979; Daniel and Derby, 1991). Crustacean predators tested as they rest in bioassay chambers have been found to increase the rate at which they flick their antennules in response to highly diluted concentrations (as low as 10^{-8} to 10^{-16} g/l) of feeding stimulants prepared from prey extracts. These same predators demonstrated locomotory responses at stimulus concentrations between 2 and 10 orders of magnitude higher than the detectable levels (Mackie and Shelton, 1972; McLeese, 1974; Pearson and Olla, 1977; Pearson *et al.,* 1979; Zimmer-Faust and Case, 1983).

Why did differences arise between the concentrations detected and those evoking locomotion? To address this question, we examined the behavioral responses of lobsters (*Panulirus interruptus*) and crabs (*Callinectes sapidus*) to prey odors: the responses were assayed while these animals were walking and while they were resting between bouts of endogenous locomotory activity.

Materials and Methods

Separate experiments were performed with spiny lobsters and blue crabs. Spiny lobsters were tested for their responses to applied chemical stimuli at the Marine Science Institute, University of California at Santa Barbara, and blue crabs were assayed at the Belle W. Baruch Marine Field Station, Georgetown, South Carolina. All specimens were captured in baited traps, then held outdoors in large circular tanks (1.5-m diameter) with continuously flowing seawater. Laboratory seawater salinity and temperature were 33‰ and 15°–18°C at U.C. Santa Barbara, and 30–32‰ and 25°–27°C at the Baruch Marine Field Station. The lobsters were fed live mussels (*Mytilus galloprovincialis*) and sea urchins (*Strongylocentrotus purpuratus*) *ad libitum;* the crabs were fed live clams (*Mercenaria mercenaria*) and oysters (*Crassostrea virginica*). Animals were always used in experiments within 3 days of capture. A single lobster or crab was put into a test tank (1.5-m diameter) without food for 24–32 h before trials commenced. The lobsters were equally represented by males and females of mean carapace length 79 ± 3 mm SD. In contrast, most of the crabs tested were male, because few females were captured; the mean carapace width was 108 mm (±6 mm SD). Each lobster or crab was used in only one trial, tattooed (Kuris, 1971), then returned to the field at least 0.5 km from the capture site.

Experiments were designed to test for effects of endogenous locomotory activity on behavioral responses to chemical stimuli. Both lobsters and crabs were presented a chemical stimulus, either as they rested or as they walked. Animals to be tested in a resting state were first observed until they had actively walked and then become quiescent. The chemical stimulus was introduced when the animals had been resting for 15–30 min. All trials were performed at night (2200–0300 h), except where otherwise noted.

To introduce chemical solutions, we attached small backpacks consisting of two thin (0.86-mm i.d.) polyethylene tubes threaded in tiny plastic guides glued onto the dorsal shell of each animal. Each tube could deliver stimuli to one side of the head. For lobsters, chemical-stimulus delivery in one tube was paired with seawater (control) input in the other tube. For crabs, we introduced the chemical stimulus—or the seawater control—into both tubes. The different methods of stimulus delivery were required because of differences in the type of response. Lobsters turn toward the site of delivery of the chemical stimulus, but crabs stop walking and probe the substratum with chelae and legs, whether the chemical stimulus is delivered to one or both sides of the head. In both lobsters and crabs, each tubing pair was supported by a single small styrofoam float at the water surface and connected to a boom and pulley system suspended above the tank. This arrangement was used to provide test animals with unrestrained access to the entire tank. Stimuli were injected at 3 ml/min into each tube, and the animals were observed for behavioral responses for 60 s. Video recording and image intensification were used to visualize lobster locomotory behavior in moonlight (see methods of Zimmer-Faust, 1987). For lobsters, a positive response was scored when an animal turned 45°–90° to one side and walked along this new bearing. The side of chemical stimulus delivery was reversed in alternate trials. Previously, we found that when identical stimuli were introduced synchronously to both sides of the head, lobsters turned equally often to their right and to their left, thus demonstrating no directional bias (Zimmer-Faust, 1993). For blue crabs, a positive response was scored when chelae and legs were used to probe the substratum. Crab behavior was directly observed in dim, far-red illumination.

Behavioral responses to waterborne substances released from natural prey were assessed. In relatively calm environments along the coast of southern California, spiny lobsters commonly travel from offshore reefs to feed on dense mussel aggregations in intertidal regions of rocky shores during flood tides (Robles *et al.,* 1990). As a test stimulus, seawater was collected 5 cm away from a large, submerged mussel bed (total area: 12 m^2). Control seawater was collected over a sand flat (0.5 m above the substratum) 15 m away from the same bed. The bed was located on a rock face at Goleta Point, within 400 m of the Marine Science Institute. This site was protected from direct wave exposure, though no attempt was made to characterize the hydrodynamic environment at the time that the seawater samples were collected. The bed was a mixed stand of *Mytilus californianus, M. galloprovincialis,* and *Septifer bifurcatus* with 2210 (±563 SD) individuals/m^2 as determined by random quadrat sampling (quadrat area: 0.01 m^2; $N = 18$ replicates). All seawater was collected in acid-washed glass containers (1-l capacity), each equipped with a glass stem and Teflon stopcock that could be turned open to allow water flow into the vessel. Seawater samples were filtered to 0.22 μm within 10 min of collection, then stored frozen in 25-ml aliquots at −20°C until used in experiments.

Lobster behavior was also assayed in response to purified adenosine 5′-triphosphate (ATP). This molecule acts as a chemical signal indicating the freshness and edibility of food and is a potent feeding attractant to spiny lobsters (Zimmer-Faust, 1993). Active and resting lobsters were each tested with ATP at 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} *M.* Lobsters at rest were tested separately during day (1100–1500 h) and night (2200–0300 h). The lobsters tested during the day resided in shelters and were at rest for 4–8 h before the trials. In contrast, lobsters tested at night had emerged from their shelters, walked for at least 10 min, and then come to rest outside the shelters for 15–30 min before trials began.

Blue crabs primarily consume infaunal bivalves (Laughlin, 1982), and are extremely voracious predators of *Mercenaria* (Castagna and Kraeuter, 1977; Sponaugle and Lawton, 1990). Juvenile clams within the size range (shell length: 1.0–2.5 cm) commonly consumed by crabs were collected from the Edisto estuary, near Charleston, South Carolina. The clams were rinsed free of mud and debris with sterile artificial seawater medium (ASW) (see Zimmer-Faust and Tamburri, 1994), then used in preparing the test solution on site. The test solution was made by bathing live clams (21.3 g of total wet tissue mass) for 2 h in 10 l of sterile ASW at the same temperature (26°C), salinity (31‰), and pH (7.8) as seawater at the collection site. A control solution was prepared identically except that fresh empty shells were used instead of live clams. Two hours after they had been added to the containers, the clams or shells were removed. The solutions were filtered to 0.22 μm, divided into 25-ml aliquots, and frozen on dry ice for transport to our laboratory at the Baruch Marine Field Station. Solutions were held in the laboratory at −76°C until used in experiments.

Results

Both crabs and lobsters commonly exhibited endogenous locomotory activity at night, but were rarely active during the day. We first performed a series of experiments at night to determine whether responses to chemical stimuli differed with activity state. While walking, both crabs and lobsters responded significantly more often to prey odor than to seawater (control) (Fig. 1; *G*-test for homogeneity: $G \geq 5.78$; df = 1; $P \leq 0.02$, both comparisons). This response consisted of either locomotion directed towards the odor source (lobsters) or substrate probing with chelae and legs (crabs). In contrast, crabs and lobsters tested while at rest responded only rarely, and the re-

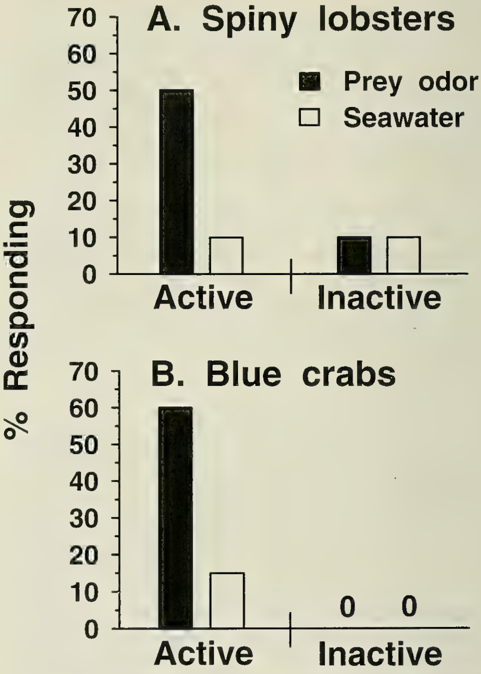

Figure 1. Percentages of (A) spiny lobsters and (B) blue crabs responding either to prey odor or seawater. Prey odor for lobsters is seawater collected near a mussel bed, whereas that for crabs is seawater conditioned by live clams (see *Materials and Methods* for details). (A) In each trial, the prey odor was introduced to one side of the lobster head and seawater to the other side. A response was scored when a lobster turned 45°–90° to either side during the first 60 s after the stimulus was introduced. Twenty trials were performed for resting (Inactive), and another 20 for spontaneously walking (Active) lobsters. (B) In each trial, either prey odor or seawater was simultaneously introduced to both sides of the crab head. A response was scored when a crab began to probe the substratum with chelae and legs during the first 60 s after the stimulus or control was introduced. A total of 80 trials was conducted, 20 each delivering only prey odor or seawater to either resting or spontaneously walking crabs.

sponses were as often to seawater as to prey odor ($G = 0.00$; df = 1; $P > 0.50$, both comparisons).

In a separate series of trials, a defined chemical stimulus (ATP) was delivered to lobsters. The ATP concentrations were log transformed, and least-squares regressions were generated to describe D/R functions. Because the D/R functions determined for lobsters tested at rest during the day and at night did not significantly differ (ANCOVA: $F = 0.02$; df = 1,3; $P > 0.50$), these data were pooled into a single group. D/R functions for resting and locomoting lobsters were then compared, and a significant difference was found (Fig. 2, and ANCOVA: $F = 98.02$; df = 1,8; $P < 0.001$). Potency analysis was performed next, after parallel D/R functions were constructed using weighted means (Tallarida and Murray, 1981). From this analysis, median effective doses (ED_{50}) of 5.9×10^{-8} *M* and 2.9×10^{-5} *M* ATP were established for behavioral responses by locomoting and resting lobsters respectively. ATP was 498 times more potent in evoking a response from active animals than from resting animals.

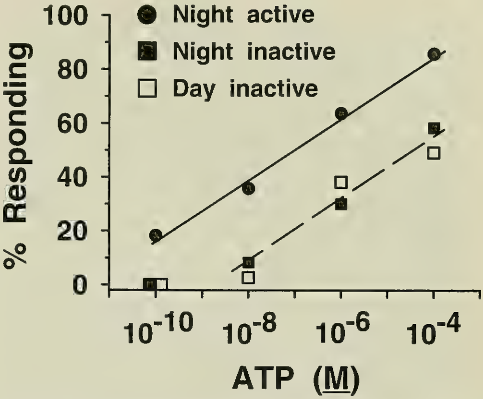

Figure 2. Percentages of lobsters responding to ATP. Between 10 and 14 trials at each concentration were performed for each of three treatments. In each trial, ATP was introduced to one side of the lobster head and seawater to the other side. A response was scored when a lobster turned 45°–90° to either side during the first 60 s after the stimulus was introduced. Of lobsters tested, ≤10% turned to the seawater side in any set of trials (data not shown). Because responses to ATP by lobsters tested at rest during the day and at night did not significantly differ (see text), these data were pooled into a single group. Least-squares regressions were then generated to describe log dose–response functions for locomoting (solid line) and resting (dashed line) lobsters.

Discussion

These results demonstrate a clear difference between the effects of prey odor on active and resting predators. Only actively walking lobsters and crabs responded significantly to prey odors. To test whether the presence or absence of a response reflected a difference in chemical sensitivity, purified feeding attractant (ATP) was introduced to spiny lobsters. The median effective dose evoking a behavioral response in resting lobsters was more than 2 orders of magnitude higher than that in active ones. Remarkably, neither the duration (15–30 min compared with 4–8 h) nor the timing (night compared with day) of the rest period influenced the sensitivity of this behavioral response in lobsters. Comparable experiments with purified feeding attractants were not performed on blue crabs, because the appropriate molecules have yet to be identified for this species.

Animal sensitivity to chemical stimuli is controlled by hormonal and peripheral and central neural mechanisms (van Drongelen *et al.*, 1978; Mankin and Mayer, 1983; Boeckh and Selsam, 1984; Ache, 1988; Derby and Atema, 1988). The integration of hormonal and neural elements in mediating olfactory perception is relatively well understood for insects (Palaniswamy *et al.*, 1979; Davis, 1984; Linn *et al.*, 1992), but not for other taxa (but see Gleeson *et al.*, 1987; Kelley, 1988; Sears *et al.*, 1991).

Environmental factors such as temperature, light intensity, photoperiod, and circadian locomotory cycle all influence chemosensitivity (Cymborowski, 1973; Baker and Cardé, 1979; Linn and Roelofs, 1986a). For example, pheromone-mediated behavior in male cabbage looper moths is strongly correlated with the production of neuromodulatory compounds. Injection of the central neuromodulator, octopamine, into male-moth hemolymph increases the sensitivity to pheromone and lowers the dose of pheromone required to initiate flight (Linn and Roelofs, 1986b). In nature, male cabbage loopers fly only at dawn and dusk when stimulated with female sex pheromone. These periods are those associated with peak octopamine production and maximum endogenous flight activity (Linn and Roelofs, 1986b).

Investigators of chemoreceptive behavior in marine organisms are faced with hard decisions. Many techniques now commonly used to increase the speed and convenience with which experiments are performed may interfere with the locomotory patterns of the test animals and thus influence the chemosensory-mediated behavior being observed. Small test chambers, Y-tubes, and other olfactometer designs can effectively regulate chemical stimulus environments, but may also reduce the walking activity characteristic of freely ranging animals.

Our own work offers a prime example of the difficult lessons to be learned. Previously, we placed lobsters in small chambers and would test individuals for their responses to ATP only if they were initially at rest (Zimmer-Faust, 1987). In fact, the threshold dose (ED_{50}) for the induction of locomotion in resting lobsters kept in those small chambers was nearly 500 times higher than the threshold for active lobsters maintained in large tanks in the experiments reported here. Based on current results, we know now that lobsters can respond to ATP at concentrations only slightly higher than those occurring naturally in seawater (10^{-9}–10^{-10} *M*; Azam and Hodson, 1977; Hodson *et al.,* 1981). Our findings do not negate the importance of the many prior studies on chemically stimulated behavior. Previous determinations may, however, have significantly overestimated the concentrations (especially thresholds) required to evoke responses whenever inactive animals were employed in assays.

Many marine predators—like spiny lobsters and blue crabs—forage only during part of the day. Crustacean, echinoderm, and fish predators, in particular, often graze on diurnal or circadian cycles (Bray and Ebeling, 1975; Nelson and Vance, 1979; Sponaugle and Lawton, 1990). We propose that the induction of locomotion by intrinsic or endogenous factors may be a widespread mechanism for bringing these predators into the vicinity of prey. We further hypothesize that predator and prey must be within the same vicinity before chemical stimuli at low concentrations will significantly modify the locomotory patterns of the predator. Low-level chemical stimuli may increase the probability of a predator contacting its prey, perhaps by inducing turning and local search or, at least, by modifying activity to keep the predator near a source of prey. Our current study shows that a predator's sensitivity to chemical stimuli can change dramatically with its activity state. This capacity seems critically important in regulating predatory behavior and in maximizing foraging efficiency.

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Detached, Purified Nerve Terminals From Skate Electric Organ for Biochemical and Physiological Studies

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Abstract. Electric organs of skate (*Raja* species) dissociate to form populations of individual electrocytes when incubated in saline solutions containing collagenase. The rate of dissociation was highly temperature dependent, with an apparent Q_{10} of >6 in the range of 6°–26°C. The number of electrocytes per organ was relatively constant and independent of electric organ size, whereas mean cell diameters increased with organ size. The activities of two cholinergic marker enzymes, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), in extracts of whole fresh organs were much less than those reported for the electric ray *Torpedo,* suggesting a lower volume of terminals in the organ. Electrocytes prepared from collagenase-treated organs had good resting potentials and generated postsynaptic evoked potentials. Spontaneous and electrode pressure-evoked miniature endplate potentials (MEPPs) were readily recorded from isolated electrocytes. Incubation periods of more than 4 days in collagenase at 6°C produced electrocytes with good resting potentials and very low MEPP frequencies, indicating denervation. Detachment of terminals and decreased MEPP frequencies were concurrent. The time course of denervation was followed with the appearance of ChAT and AChE activities in a small particulate fraction derived from washed electrocytes. Peak activities of both enzymes were seen at 4 days of incubation at 16°C, but after 20 h at 16°C. Electrocytes from 4-day, 6°C incubations showed detached, mitochondria-rich nerve terminals and dissociated Schwann cells. In unfixed preparations examined with Nomarski optics, isolated nerve terminals were recognized and distinguished from nucleated Schwann cells. Electron micrographs show that isolated terminals were similar to attached terminals just before they dissociated. The MEPP frequencies and evoked potentials were normal at terminals just before dissociation.

We conclude that the transmitter release process was normal in detached terminals and in terminals free of Schwann cells.

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Introduction

The electromotor system of *Torpedo* is a well-established model for studies on the chemistry of cholinergic synaptic transmission (for review see Whittaker, 1992). Several other electric fish (*e.g., Electrophorus, Malapterusus,* and *Raja*) have comparable organs but, for biochemical studies, these have received little attention. The cholinergic nature of transmission in skate (*Raja*) electric organ was established by means of microelectrode techniques (Brock *et al.,* 1958; Bennett, 1961) and enzyme histochemistry of AChE (Couteaux, 1963). More recently, quantal release of transmitter from these terminals was shown to be similar to that found at mouse and frog neuromuscular junctions (Kriebel *et al.,* 1987, 1988). In common with these neuromuscular synapses, but unlike the neuroelectrocyte synapses in *Torpedo,* the synaptic vesicles in skate are homogeneous in size (Fox *et al.,* 1988). Innervated electrocytes that retain electrical responsiveness to presynaptic stimulation can be isolated from skate electric organ following collagenase treatment at room temperature (Fox *et al.,* 1990). These authors also showed that extended exposure of the organ to collagenase results in denervation of electrocytes and formation of clumps of detached nerve terminals encapsulated by Schwann cells. We have used these observations as a starting point for the present study, in which we combined biochemical measurements with microscopy and electrophysiology to further investigate the innervation of skate electric organ and to monitor in greater detail the collagenase-induced detachment of nerve terminals. We report here the conditions that produce functional electrocytes and detached nerve terminals freed from adherent Schwann cells. A preliminary report of some of this work was presented at the General Scientific Meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts (Dowdall *et al.*, 1989).

Materials and Methods

Animals

Most experiments were done using adult specimens of *Raja erinacea* supplied by the Marine Resources Department of the Marine Biological Laboratory in Woods Hole. These varied in maximum width from 16.5–37 cm. Fish were killed with a blow to the head. Tails were amputated and electric organs removed by blunt dissection. In some experiments, specimens of *R. naevus* (cuckoo ray) were used. These were obtained from the Gatty Marine Station, St. Andrews, Scotland, and maintained in aquaria at 15°C in Nottingham, England.

Treatment of electric organs

Dissected organs were placed in plastic petri dishes and immersed in skate saline of the following composition (m*M*): NaCl (280), KCl (12), $CaCl_2$ (10), $MgCl_2$ (2), urea (360), glucose (1), and HEPES buffer (10) pH 7.3. Measurements were made of the total length and wet weight of organs. Additionally, single transverse sections of 5 mm were removed from the middle of one of each pair of organs; after being weighed, these were used to determine the number of electrocytes in the organ. Counting of dissociated electrocytes was possible after they had been incubated overnight in 1% (w/v) collagenase at room temperature. Wells of a plastic macrophage migration plate covered with glass coverslips were used for incubation chambers. In some experiments the diameter of electrocytes was determined by aligning cells edge to edge over a 2-mm^2 reticle.

Total tissue homogenates for measuring enzyme activities were prepared from organs that were snap-frozen in liquid nitrogen, then reduced to powder. Tissue powders were extracted with 50 m*M* phosphate buffer (pH 7.4) containing 300 m*M* NaCl, 20 m*M* EDTA, and 0.5% (w/v) Triton X-100; a Teflon–glass homogenizer was used to produce 10% (w/v) homogenates.

Bulk electrocyte preparations were produced by incubating whole electric organs in an equal volume of skate saline containing 1% (w/v) collagenase (Worthington CLS I) for up to 7 days at 6°C. For comparison, higher temperatures were also used in some experiments. In all cases the incubation vessels (normally 35-mm culture dishes) were left undisturbed throughout the incubation. Collagenase dissolved in skate saline contained insoluble material, and this was removed by centrifugation in a microfuge and gel-filtration through a Sephadex PD 10 column. The collagenase-containing fraction (brown) was recovered and adjusted where necessary to 1%. Collagenase-treated organs were dispersed by gentle aspiration of material and medium and expulsion with plastic transfer pipettes (10 cycles).

Dissociated electrocytes were washed free of smaller cellular material (fragments of electrocytes and nerve fibrils, glia and nerve terminals) by sequential washing and sedimentation at unit gravity in polypropylene centrifuge tubes at 0–4°C. Tissue suspensions (~20% w/v) in skate saline formed loose pellets of electrocytes when left to settle for 3 min. The supernatant was gently removed, retained, and replaced by an equal volume of fresh saline. Following resuspension of the loose pellet, this washing procedure was repeated 3–4 times, with all washings being combined with the initial supernatant. Particulate material was concentrated from the supernatant fractions by centrifugation at 17,000 g_{av} for 20 min with a Sorvall SS-34 rotor. The resultant pellet (fraction $P_{1,2}$) was further fractionated into large (P_1) and small (P_2) particulate material by differential centrifugation in a microfuge (Sorvall Microspin 24S) for 1 min at 3000 rpm followed by 5 min at 13,500 rpm. Fraction P_1 contained fragments of electrocytes and nerve fibrils together with some smaller particles. None of this larger debris was seen in fraction P_2 which was therefore used as the crude nerve terminal preparation for subsequent biochemical analyses.

Morphological techniques

Unfixed samples of dissociated electrocytes in saline were viewed with standard light microscopy at low (binocular dissecting microscope) and high (Zeiss Axiophot) magnification. Nomarski and phase optics were used at the higher magnifications to identify detached nerve terminals. For electron microscopy, samples were fixed in 2.5% glutaraldehyde, 10% sucrose (0.292 *M*) in 0.2 *M* sodium cacodylate buffer pH 7.4 at 4°C for 12–24 h. Primary fixation buffer was removed and samples were washed in fresh buffer prior to post-fixation in 1% osmium tetroxide in 0.4 *M* sodium cacodylate and *en bloc* staining with 1% uranyl acetate in 0.25 *M* sodium acetate buffer pH 5.5. Ethanol, propylene oxide, and Epon 812 were used throughout as dehydrating, transferral, and embedding agents.

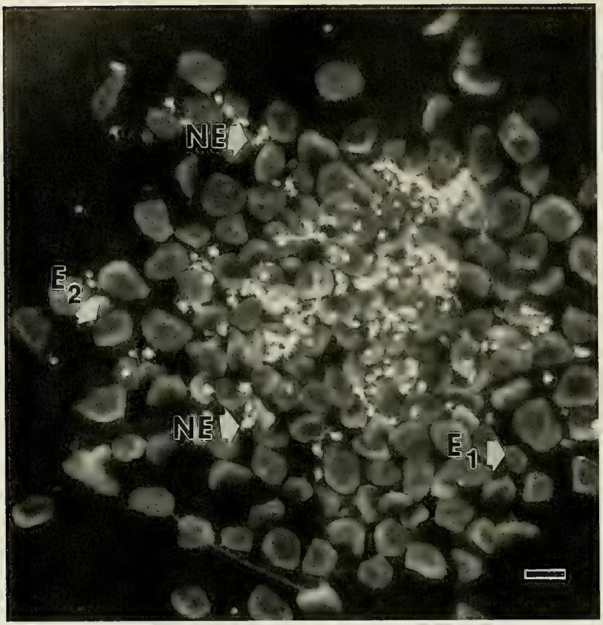

Figure 1. Dissociated electrocytes from *Raja erinacea* after low-temperature incubation with collagenase. Note the range of sizes of these whole, unfixed electrocytes as seen in the dissecting microscope. Electrocyte E_1 is about one-third the size of E_2. NE = nerve ending cluster. Bar = 0.4 mm.

Enzyme assays

Lactate dehydrogenase (LDH) was measured by following the oxidation of NADH spectrophotometrically (Johnson, 1960) in a final volume of 1 ml. Acetylcholinesterase (AChE) was measured using a final incubation volume of 1 ml (Ellman *et al.*, 1961). Choline acetyltransferase (ChAT) was used as a marker for nerve terminal cytoplasm and was measured by means of a radiometric assay described by Fonnum (1975), with the following modifications. The final incubation volume of 10–100 μl contained NaCl (300); EDTA (20); choline chloride (10); acetyl CoA (0.2); eserine sulfate (0.15); sodium phosphate buffer, pH 7.4 (50); 0.5% Triton X-100; and ^{3}H- or ^{14}C-labeled acetyl CoA (500 nCi/ml). Incubations were at 26°C for time periods up to 2 h, and three time points were used for each determination. Reactions were terminated by transfer of aliquots (5–50 μl) to mini-scintillation vials containing 1.6 ml of 10 m*M* phosphate buffer and 0.67 ml acetonitrile containing sodium tetraphenylboron (10 mg/ml). Labeled ACh was measured by liquid scintillation after addition of 3.5 ml of water-immiscible scintillation fluid, gentle shaking, and phase separation. Labeled acetyl CoA remained in the lower (aqueous) layer and therefore made no contribution to the count rate. This procedure gave the same results as one in which the volumes of reagents after termination were 3 times greater (as originally described by Fonnum, 1975). Both collagenase and skate saline were found to inhibit ChAT activity when added to extracts of whole electric organ. This inhibitory effect was therefore minimized by resuspension of pelleted fractions in ice-cold 0.45 *M* NaCl followed by a brief centrifugation in a microfuge. This washing procedure was repeated prior to enzyme assay.

Electrophysiology

Dissociated electrocytes were bathed in saline and viewed with a dissection microscope. Excitatory miniature endplate potentials (MEPPs) and evoked potentials (EPPs) were recorded with standard microelectrode techniques. EPPs were produced with supramaximal stimulation with either a suction electrode or field stimulation. For suction electrode stimulation, the surface of electrocytes was drawn against the suction electrode tip. Field stimulation was achieved by placing Ag-AgCl wires along the sides of the bath.

Electrocytes show few, if any, spontaneous MEPPs, and intracellular MEPPs are very small (Kriebel *et al.,* 1987). MEPP frequencies and amplitudes were increased by pushing the electrode against the cytoplasmic surface to record focally. The MEPPs studied here are "pressure evoked." Healthy nerve endings are indicated by low MEPP frequencies and sensitivity to electrode pressure such that frequencies are reversibly increased as the electrode is advanced. The condition and density of endings during collagenase treatment were evaluated from the response of the terminal to electrode pressure, which induced MEPPs, and by field stimulation, which generated EPPs.

Results

Electrocyte numbers and size

Skates have spindle-shaped electric organs that are slightly flattened dorsoventrally. In the present study, individual organs of *R. erinacea* varied from 9–22 cm in length and from 2.5–5.0 cm in maximum diameter. Organ wet weight varied from 116–1246 mg (mean 553 ± SEM 47; n = 34 fish) and was positively correlated with fish size. Electrocyte numbers per organ varied between 1.1 and 6.2×10^3, with a mean of 2.5 and an SEM of $\pm 0.19 \times 10^3$, $n = 28$. The mean number for *R. erinacea* is therefore essentially the same as that of 2.4×10^3 for *R. clavata* (Brock *et al.,* 1953). The small coefficient of variance demonstrates that the number of electrocytes was independent of organ size. Thus growth of electric organs is due to the increase in size of individual electrocytes. This conclusion is borne out by the mean electrocyte diameters, which varied from 0.25 to 0.65 mm for organ weights of 116 to 800 mg. Dissociated electrocytes as seen at low magnification are illustrated in Figure 1.

Viability and temperature studies

Viability studies of electrocytes in dissected organs were carried out with *R. naevus* and *R. erinacea. R. naevus:* Organs were stored in saline at 6°C for periods up to 18 days and small (1–2 cm) sections removed as required. Penetration of intracellular electrodes into electrocytes was greatly facilitated by prior treatment of the tissue with collagenase. This resulted in the partial dissociation of the loosely packed electrocyte columns into smaller pieces in which individual electrocytes were attached to the fine branches of common efferent nerves. The higher temperatures were detrimental to electrocyte resting potentials and also produced sticky preparations. Temperatures of 30°C or lower produced electrocytes with healthy resting potentials (~−60 mV). The most stable electrocytes were produced by extended incubation periods (11–18 h) at 6°C. When these conditions were used, the physiological state of electrocytes *in vitro* was remarkably stable. Resting and evoked potentials were essentially unchanged even after 11 days *in vitro* (Fig. 2). After 14 days electrocytes gave smaller responses, and at 20 days no viable cells could be found.

Biochemical measurements

Nachmansohn and Meyerhof (1941) and, more recently, Fox *et al.* (1990) determined the ACh content in skate electric organ. We therefore determined the activity of two cholinergic enzymes (ChAT and AChE) in extracts of whole organ and in fractions prepared from collagenase-digested organ. The activity of a general cytoplasmic enzyme, lactate dehydrogenase (LDH), was also determined as a measure of overall cellular density.

Both AChE and ChAT activities were tested for their sensitivities to established enzyme inhibitors. BW 284 c51 (a selective inhibitor) inhibited AChE activity completely at 1 m*M* and by 50% at 2 n*M*. Therefore, any contribution from butyrylcholinesterase to the measured activity was negligible. Bromoacetylcholine, a specific inhibitor of ChAT (Tuček, 1982), was an effective inhibitor in the skate extracts (50% inhibition at 10 n*M* and 95% at 1 m*M*). A sensitivity comparable to that of this inhibitor has been reported for ChAT from *Torpedo* electric organ (Eder-Coli and Amato, 1985).

Enzyme activities are given in Table I together with the corresponding values for *Torpedo,* some unpublished;

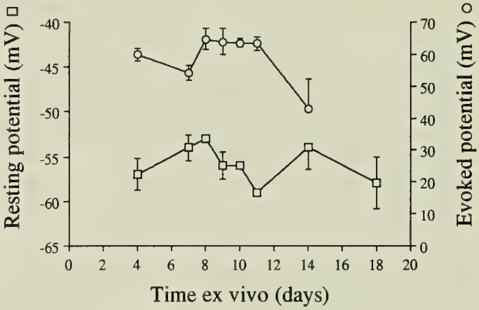

Figure 2. Stability of the electrophysiological state of electrocytes *in vitro.* Results from dissected organ of *Raja naevus* maintained in saline at 6°C for the times shown. Resting and evoked potentials were measured using intracellular microelectrodes on electrocytes prepared from small pieces of organ exposed to collagenase for 12–18 h at 6°C. Resting potentials are from individual cells or are means ± range (n = 2) or SD (n = 3–6). Evoked potentials are from individual cells stimulated with a train of pulses at 1 Hz; mean response amplitudes are from pulses 1, 2, 4, and 7 (± SD).

Table I

Biochemical characteristics of skate and Torpedo *electric organs*

			Skate			
Activity	Units/g	Torpedo	Mean	SEM	*n*	%
ChAT	nmol/h	7500[a]	235	26	11	3.1
AChE	μmol/min	137–274[a]	12.5	1.5	10	9.0–4.5
ACh	nmol	1475[b]	48.6[c]	—	—	3.3
LDH	μmol/min	10.5[d]	2.82	0.25	10	26.8

Enzyme activities for skate are from the present study. Other values are from the literature as follows: [a] Dowdall (1977); [b] Volknandt and Zimmerman (1986); [c] Fox *et al.* (1990); [d] Dowdall (unpub. data). In the final column, skate values are expressed as a percentage of those of *Torpedo.*

values for ACh from both organs are shown for comparison. The activities of all three enzymes were much lower in skate than in *Torpedo,* and the results for ChAT, in particular, closely parallel the previously reported concentrations for ACh (Fox *et al.,* 1990). AChE activity was comparable to that previously reported for *R. undulata* (Nachmansohn and Meyerhof, 1941). Taken together, these findings show that the terminal volume in skate electric organ would be 3%–5% of that in *Torpedo.* If allowance is made for the lower cellular density in skate and the larger volume of individual electrocytes (as indicated by LDH activity), then the terminal volume of skate electric organ is between 10% and 18% of that found in *Torpedo.*

Denervation by collagenase action

The action of collagenase on skate electric organ has two distinct, but partially overlapping, phases: first, the dissociation of innervated electrocytes; and second, the denervation of individual electrocytes and the concomitant release of nerve terminals into the surrounding fluid (Fox *et al.,* 1990). Biochemical markers (ChAT and AChE) were used to follow the time course of this denervation. A simple fractionation scheme was devised to separate detached nerve terminals from the much larger electrocytes (see Materials and Methods).

The activities of ChAT and AChE in the small particulate fraction from collagenase-treated electric organ are shown as a function of incubation time and temperature in Figure 3. At 6°C the dissociation of the electric organ was very much slower than at room temperature, requiring days rather than hours to run its full course. AChE activity was detectable after 1 day but required a further 3 days to reach a maximum, thereafter declining. The appearance of ChAT activity in the small particulate fraction was broadly similar to this pattern, sharing the same peak at 4 days but with a more marked lag period. The loss of both activities after 4 days suggests lower yields of nerve terminals after extended periods of collagenase digestion.

The difference in tissue dissociation rates at 6°C (this study) and 26°C (Fox *et al.,* 1990) was surprisingly large (~40-fold) and much greater than the 6.3-fold expected for a single enzyme reaction with a Q_{10} of 2.5. The observed temperature dependence is, however, consistent with cooperative enzyme action. Collagenase, by itself, is known to be ineffective for total tissue dissociation and is therefore used in conjunction with other proteases. The collagenase preparation from *Clostridium* is not highly purified and contains sufficient quantities of contaminating proteases to effect tissue dissociation. The time course of denervation at 16°C (Fig. 3B) was 5 times faster than at 6°C, as judged by the rate of accumulation of AChE and ChAT activities in the small particulate fraction

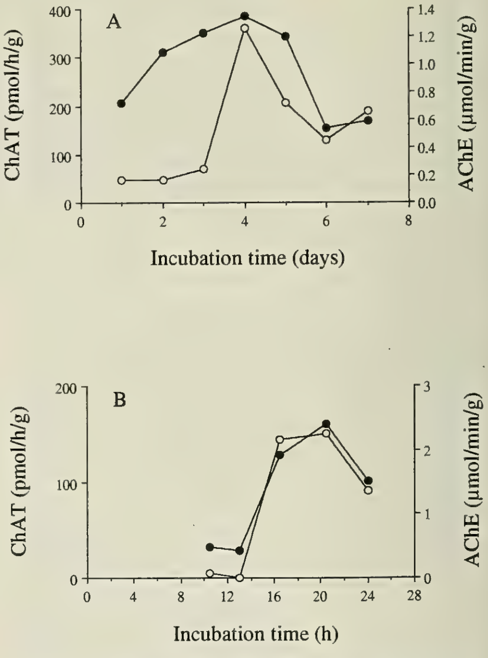

Figure 3. Detachment of nerve terminal marker enzymes from electrocytes by collagenase. Whole organs were incubated in collagenase for time periods varying between 10 h and 7 days and, where possible, were washed free of incubation fluid before tissue dispersion. Small particulate material (fraction P_2) was collected from washed electrocytes as described in the Materials and Methods and assayed for ChAT (open circles) and AChE (closed circles). Activities are expressed per gram wet weight of organ. (A) Time course at 6°C showing peak activities at 4 days. (B) Time course at 16°C showing accelerated rate of detachment and parallel behavior of both activities up to 24 h.

(peaking for both at 20.5 h). In parallel with these changes, soluble AChE activity progressively increased in the incubation fluid up to 20.5 h, thereafter declining (see legend to Fig. 3). At its maximum this was some 32% of the AChE activity of whole tissue and presumably arises from the action of collagenase on collagen-tailed isoforms of this enzyme. In *Torpedo* these forms constitute 40% of total AChE; the hydrophobic (6S) form, localized in the presynaptic membrane, accounts for the other 60% (Li and Bon, 1983). It seems likely that the AChE activity of fraction P_2 from skate electric organ corresponds to the 6S form in *Torpedo.*

Comparison of enzyme activities in fraction P_2 with those measured for total tissue suggests a very poor recovery of nerve terminals in this crude fraction. ChAT activity was only 0.05%–0.15% of total tissue, whereas AChE was 10%–20%. The large discrepancy between these figures is almost certainly due to the problem of measuring ChAT in extracts prepared from collagenase-treated tissue (see Enzyme assay section in Materials and Methods). It is possible that the nerve terminals were seriously damaged by the washing technique used to remove traces of collagenase. AChE activity is therefore a more reliable indicator of nerve terminal recovery than is ChAT.

The morphology of electrocytes after treatment of organs for 4 days at 6°C is shown in Figures 4 and 5. At this stage, the dissociation of electrocytes from innervating nerves is almost complete, with the latter having a "balled-up" appearance that we refer to as a nerve-ending cluster (NE) in Figures 2 and 4. With Nomarski optics (Fig. 4), we see clusters of isolated nerve endings. Separation of nerves from electrocytes leaves some nerve terminals attached to the electrocyte surface, as revealed by the electron micrograph shown in Figure 5. The terminals are 1–3 μm in diameter and are filled with electron-lucent synaptic vesicles together with somewhat larger clear vacuoles and numerous intraterminal mitochondria.

The most striking feature of the detachment process at 6°C was the dissociation of Schwann cells from the terminals. In contrast, at room temperature the Schwann cells creep over newly exposed surfaces of nerve terminals and eventually encapsulate detached terminals (see fig. 15 of Fox *et al.,* 1990). Conceivably, the lower temperature inhibits the motility of the Schwann cells and thus prevents encapsulation. When salines from washed electrocytes are examined with Nomarski optics, free nerve terminals can be identified (Fig. 6). Detached Schwann cells have quite a different appearance because of their single large nuclei.

Electrophysiological studies

Previous physiological studies on transmission at the neuro-electrocyte junction of skate were carried out on freshly dissected tissue, either when it was in an intact, untreated state (Kriebel *et al.,* 1987) or after it had been briefly exposed to collagenase at room temperature (Fox *et al.,* 1990). In view of the greatly extended periods of exposure to collagenase used in the present study, it was important to establish the basic electrophysiological properties of electrocytes so treated. Accordingly, electrocytes dissociated by 6°C incubations were checked by using extracellular and intracellular microelectrodes. Dissociated electrocytes produced endplate potentials of 50–100 mV when stimulated repetitively at 2 to 10 Hz with suction electrodes applied to innervating nerves (up to 4 days) or the innervated external surface of electrocytes (4–7 days). For incubation periods up to and including 4 days, MEPPs at normal, albeit low, frequencies were obtained. As in previous studies with fresh tissue, MEPP amplitudes of two size classes were detected (Kriebel *et al.,* 1987, 1988). Histograms of MEPP amplitude distributions show two distinct classes. The larger class has a bell-shaped distribution, so these are referred to as bell-MEPPs; the smaller class has a skewed distribution, so these are referred to as skew- or sub-MEPPs (Fig. 7, traces A and C_1). The intracellularly recorded MEPPs in Figure 7 are focally recorded and are in the millivolt range. When the electrode tip was in the middle of the electrocyte, MEPPs were less than 100 μV because of the low input impedance. Therefore, truly intracellular recordings may not show MEPPs, which could be lost into the noise. We were always careful to push the electrode against either the inside or outside surface of the electrocyte when eval-

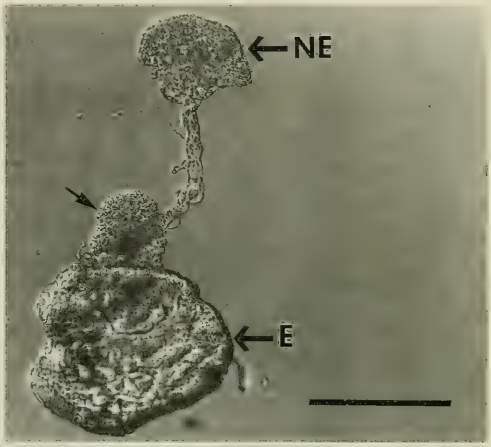

Figure 4. Light micrograph of an isolated, unfixed nerve-ending cluster (NE) under Nomarski optics. Note that another branch of the nerve (small arrow) is still partially "attached" to the electrocyte (E). Bar = 0.4 mm.

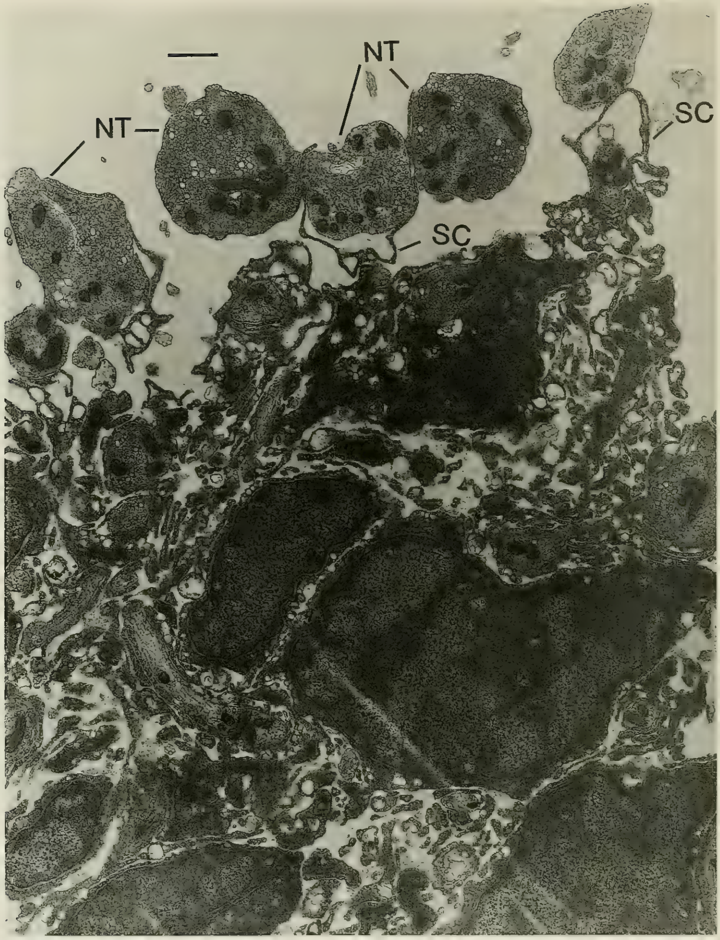

Figure 5. Detachment of nerve terminals from the innervated surface of an electrocyte. Transmission electron micrograph of washed electrocyte fraction following 4 days collagenase treatment at 6°C. Nerve terminals (NT) are seen at the innervated surface at various stages of detachment. Associated Schwann cells (SC) appear to separate from terminals, leaving some of them completely free. Note the presence of many intraterminal mitochondria. Calibration bar = 1 μm.

uating the physiological condition of the terminals. We usually used the cytoplasmic surface because K^+Cl^- leakage would not increase the MEPP frequency (Kriebel *et al.,* 1988). In contrast, potassium ion leakage from an extracellularly placed electrode increased MEPP frequency. The K ion effect was independent of the pressure (deformation) effect that resulted from pushing the electrode against the cytoplasmic surface. MEPP frequencies were generally low but increased markedly in response to slight pressure from the recording electrode against the innervated surface of the electrocyte membrane (Fig. 7, trace C_2). After 6–7 days of treatment, spontaneous MEPP frequencies were markedly lowered, but pressure-evoked MEPPs could be elicited. The fall in MEPP frequency between 4 and 6 days corresponded to the advanced state of denervation and agreed with biochemical observations.

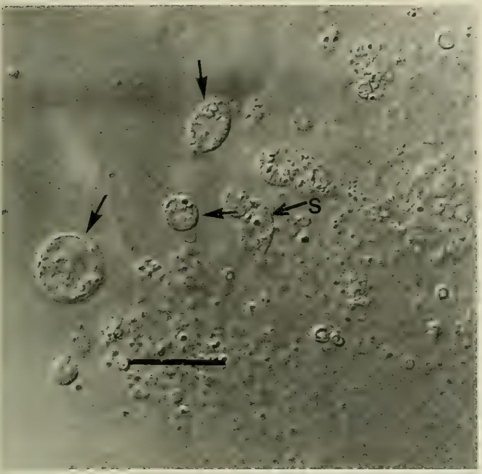

Figure 6. Light micrograph of isolated, detached nerve terminals (arrows). Unfixed washed electrocytes after collagenase treatment at 6°C for 4 days were resuspended in saline and examined with Nomarski optics. Loosely adherent nerve terminals become detached from the electrocytes and are seen as free-floating structures. They are readily identifiable by their size and content of intraterminal organelles of mitochondrial dimensions (see Fig. 5). A nucleated Schwann cell (arrow and S) is identified by the large nucleus. Calibration bar = 0.2 μm.

Discussion

In the present study we show that skate electric organs dissociate into their component cellular elements (electrocytes, Schwann cells, nerves, and nerve terminals) when incubated in saline solutions containing 1% collagenase at temperatures down to 6°C. In contrast, we have not been able to dissociate *Torpedo* nerve endings from their electrocytes. The maintenance of skate tissues at these lower temperatures has several important advantages. It is more physiological, because skates prefer cold water; it results in very stable electrocyte preparations (see Fig. 2); and it inhibits the movement of the Schwann cells and thus prevents encapsulation of detached nerve terminals during collagenase-induced denervation of dissociated electrocytes (Figs. 5 and 6). Nerve terminals prepared using collagenase at 6°C are therefore purer and probably in a better physiological state than corresponding preparations derived from tissue incubated at room temperature. The disadvantage of using lower temperatures is the relatively long digestion period. However, as judged by electrocyte viability, this does not compromise the physiological quality of subsequent preparations. Use of temperatures between 6° and 16°C might accelerate the digestion somewhat without compromising purity or physiological state, but this would require testing.

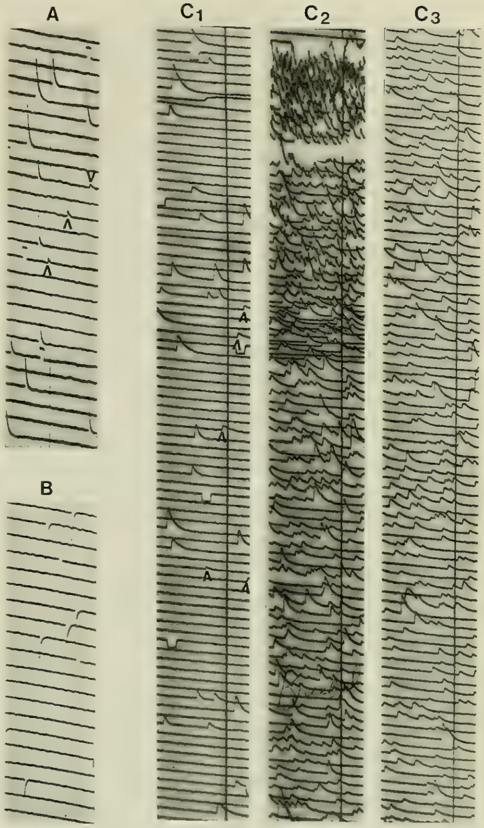

Figure 7. Miniature end-plate potentials (MEPPs) from collagenase-dissociated electrocytes. The electrocytes have detached from the nerve fibers after 4 days at 6°C, but the remaining nerve terminals still generate MEPPs. (A) Intracellular focal recording from the cytoplasmic innervated surface. This low frequency of spontaneous activity is similar to control records from untreated tissue (see Kriebel *et al.,* 1987). Note the presence of some MEPPs with very small amplitudes (arrowheads) which belong to the sub-MEPP class described previously (ref. as above). Calibration signals are 20 ms; traces are 200-ms long, intertrace distance = 1 mV. (B) Focal extracellular recordings. Calibration as in A. (C) Pressure-evoked burst of MEPPs recorded focally from the cytoplasmic surface. The recording electrode is advanced against the cytoplasmic surface of the electrocyte membrane to elicit a pressure-evoked increase in spontaneous MEPP frequency. (1) Activity recorded as the electrode touches the cytoplasmic surface. Arrowheads indicate sub-MEPPs as seen in A. Calibration signals are 1 ms: traces are 10 ms long, intertrace distance = 1 mV. (2) The electrode has been advanced against the cytoplasmic surface. The top of the film strip is the start of the pressure-induced burst. (3) Two minutes after 2. Note that MEPP frequency has decreased but remains higher than just prior to burst.

A notable feature of the detached nerve terminals described in this paper is that they are distinguishable from Schwann cells so that they can be patch clamped. Preliminary studies suggest the presence of the A current of K channels (D. R. Matteson, pers. com.) and a calcium channel similar to the Q sub-type (Richardson *et al.,* 1995). With this preparation, presynaptic ion channel physiology can be investigated on isolated cholinergic nerve terminals without recourse to manipulations such as fusion (Umbach *et al.,* 1984; Edry-Schiller *et al.,* 1991).

The detached nerve terminals from skate electric organ deserve comparison with "synaptosomes," which are formed by homogenizing *Torpedo* electric organ. In both cases the tissue disruption techniques employed lead to the dissociation of presynaptic terminals from the innervated surface of the electrocytes. With *Torpedo,* these detached terminals can be bulk-isolated by conventional subcellular fractionation and are recoverable in synaptosome fractions: such preparations have been widely used for biochemical studies (see Dowdall and Zimmermann, 1977; Michaelson and Sokolovsky, 1978; Morel *et al.,* 1977; and Zimmermann *et al.,* 1979). The % yield of *Torpedo* synaptosomes is very low (reviewed by Whittaker, 1992) but reasonable quantities can be isolated because the organs are large and very richly innervated. With skate, the bulk purification of detached nerve terminals is likely to prove much more difficult to achieve because of the limited numbers of terminals available. Thus even if the % yield of detached nerve terminals is higher than in *Torpedo,* this is unlikely to compensate for the much smaller size and lower density of innervation of the organs.

Despite this limitation, the skate electric organ nerve terminals are still attractive subjects for physiological studies. In detached form, they have several features that are essential for the application of patch-electrode techniques: size; accessibility; and the absence of adherent, encapsulating Schwann cells. So far the use of patch electrodes on vertebrate nerve terminals has been possible only with isolated peptidergic endings of 1–10 μm diameter from rat neurohypophysis (Lemos and Nowycky, 1989) and with the giant, cholinergic, calyx synapse of the chick ciliary ganglion (Stanley, 1991). Nerve terminals with diameters up to 5 μm are present in the electric organs of *Raja clavata* and *R. montagui* (Richardson *et al.,* 1995), and these might provide an opportunity to examine dynamic aspects of presynaptic channel activity directly in their normal environment, rather than in isolated synaptosome preparations (Umbach *et al.,* 1984; Edry-Schiller *et al.,* 1991). A "nerve plate" preparation consisting of a plexus of nerve fibers with associated Schwann cells and nerve fibrils with attached terminals (equivalent to the nerve-ending clusters described in this study) can be isolated from skate electric organ following collagenase treatment. It is encouraging for prospective studies that functional voltage-sensitive calcium channels have been detected in the nerve terminals of these preparations with the use of microfluorimetric imaging techniques (Richardson *et al.,* 1995).

The electrophysiological finding that the evoked response is "normal" up to the dissociation condition at which the terminals detach strongly indicates that the process of transmitter release is physiologically normal in skate isolated terminals. This conclusion is further supported by the similarity in the ultrastructure of nerve endings before and after detachment.

Acknowledgments

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Retinal Anatomy of a New Species of Bresiliid Shrimp From a Hydrothermal Vent Field on the Mid-Atlantic Ridge

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Abstract. A new species of shrimp (*Rimicaris* sp.) was recently collected from the Snake Pit hydrothermal vent field on the Mid-Atlantic Ridge. Until the discovery in 1989 that the deep-sea, hydrothermal vent species, *Rimicaris exoculata,* possessed a hypertrophied dorsal eye, everyone believed that animals recovered from vent environments were blind. Like *R. exoculata, Rimicaris* sp., a small orange bresiliid shrimp, has an enlarged dorsal eye specialized for detecting light in a very dim environment instead of the expected compound eye. The individual lenses characteristic of a compound eye adapted for imaging have been replaced in *Rimicaris* sp. by a smooth cornea underlain by a massive array of photosensitive membrane. The number of ommatidia in this species is about the same as in shrimp species that live at the surface; however, the photoreceptors are larger in the deep-sea species and the shape of the photoreceptors is markedly different. The light-sensitive region of the photoreceptor is much larger than those of other shrimp and the rest of the receptor is much smaller than normal. All screening pigment has moved out of the path of incident light to a position below the retina, and the reflecting pigment cells have adapted to form a bright white diffusing screen between and behind the photoreceptors. The ultrastructure of the microvillar array comprising the rhabdom is typical for decapod crustaceans; however, there is a much greater volume density of rhabdom (80% to 85%) than normal. There is no ultrastructural evidence for cyclic rhabdom shedding or renewal. *Rimicaris* sp. has apparently adapted its visual system to detect the very dim light emitted from the throats of the black smoker chimneys around which it lives.

Introduction

In 1977, hydrothermal vents were first directly observed on the ocean floor along the Galapagos Ridge (Corliss *et al.,* 1979). Additional active hydrothermal fields were discovered along spreading centers and back-arc basins in the Pacific and along the Mid-Atlantic Ridge (see Tunnicliffe, 1992, for a review). In 1986, the first two hydrothermal vent sites on the Mid-Atlantic Ridge (TAG Site and Snake Pit Site, Rona *et al.,* 1986) were visited with the deep submergence vehicle (DSV) *Alvin.* Two more active vent fields (Lucky Strike Site and Broken Spur Site) were first visited with DSV *Alvin* during R/V *Atlantis II* cruise 129 in the early summer of 1993. Figure 1 shows the location of these four hydrothermal vent fields. More sites are likely to be discovered as exploration of the Mid-Atlantic Ridge continues.

Unlike the Pacific vents, where vestiform tube worms and bivalves dominate the biota, Mid-Atlantic Ridge vents have major populations of bresiliid shrimp, including many new species. Two of these species, *Rimicaris exoculata* (Williams and Rona, 1986) and *Rimicaris* sp., live in massive swarms on the sides of black smoker chimneys. Both species have large white structures on the anterior

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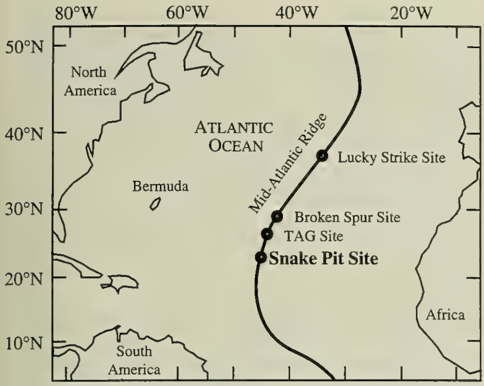

Figure 1. Map showing the Snake Pit hydrothermal vent field, where *Rimicaris* sp. was collected, and the other known hydrothermal sites along the Mid-Atlantic Ridge. The Snake Pit field is 3500 m below the surface at 23°20.3′N; 45°0.5′W. The TransAtlantic Geotraverse (TAG) field is 3700 m below the surface at 26°8.3′N; 44°49.5′W. The Broken Spur field is 3100 m below the surface at 29°10.0′N; 43°10.4′W. The Lucky Strike field is 1700 m below the surface at 37°17.6′N; 32°16.5′W.

part of their dorsal surfaces, and in *R. exoculata* this structure has been shown to be an eye (Van Dover *et al.*, 1989; O'Neill *et al.*, 1995).

The discovery of an unusual and large eye on a hydrothermal vent species which lives 3500 to 3700 m below the ocean surface implies a heretofore unrecognized source of light. Previous to the discovery of the dorsal eye of *R. exoculata,* everyone assumed that, since no light reached hydrothermal vent fields from the surface, animals there lived in complete darkness (*e.g.*, Tunnicliffe, 1992). Pelli and Chamberlain (1989) calculated that *R. exoculata* ought to be able to detect black-body radiation from the tops of the black smoker chimneys that are gushing 350°C vent water. The light emitted from the tops of black smokers has now been photographed, although the mechanism by which it is produced is still uncertain (Van Dover *et al.*, 1994).

We undertook R/V *Atlantis II* cruise 129-7 in May 1993 to investigate vision in hydrothermal vent shrimp more thoroughly. During the cruise, we realized that the small orange bresiliid shrimp we were collecting from the Snake Pit Site was very likely a distinct species. Individual live specimens survived well at the surface for many days, and, as a result, we achieved preliminary electrophysiological investigations of nerve fiber responses from the antennal nerves to tactile and chemosensory stimuli (Renninger *et al.*, 1994, 1995). Here we present the structure and ultrastructure of the dorsal eye of the new, unnamed orange *Rimicaris* species from the Snake Pit Site. Two abstracts that mention this work have appeared (Chamberlain *et al.*, 1994a,b).

Materials and Methods

Animals

Specimens of *Rimicaris* sp. for morphological study were collected on R/V *Atlantis II* cruise 129-7 during DSV *Alvin* dives #2613, #2618, and #2623 at the Snake Pit Site. The shrimp were collected with one of *Alvin's* mechanical arms, which sucked shrimp into a transparent chamber with a hose connected to a hydraulic pump. Specimens were brought to the surface and some were immediately dissected and fixed for morphological study while others remained alive in 10°C seawater for over 2 weeks for electrophysiological investigations. Live animals were examined and compared with other vent shrimp with a Nikon SMZ-10 (Garden City, New York) stereomicroscope.

Fixation and histology

Under conditions of ambient light on board the R/V *Atlantis II,* live animals were coarsely chopped and fixed by immersion in a solution of 0.8% glutaraldehyde, 5% paraformaldehyde, 4.5% sucrose, and 3% NaCl in 0.1 *M* Sorensen's phosphate buffer at pH = 7.2. After 18 h at 4°C in darkness, the central compartment of the dorsal carapace between the gills was dissected free from the anterior margin posteriorly to include both wings of the dorsal eye. The tissue was then washed in phosphate buffer with 8% sucrose and postfixed with 1% OsO_4 in phosphate buffer with 8% sucrose for 1 h at room temperature. Thereafter, it was rinsed in distilled water and dehydrated through a graded ethanol series to absolute ethanol. Finally, the tissue was transferred to propylene oxide, infiltrated with a series of propylene oxide/epoxy resin mixtures, rotated overnight in pure epoxy resin, and embedded in Epon-Araldite blocks for light and electron microscopy. For a discussion of alternate collection and fixation strategies see O'Neill *et al.* (1995).

The embedded tissue was taken to Syracuse University where serial 1-μm sections and selected gold and silver thin sections were cut using glass knives and a Sorvall MT2-B ultramicrotome (Dupont-Sorvall, Newtown, Connecticut). Sections were cut either in a plane tangential to the surface of one wing of the eye or in a plane perpendicular to that surface.

The 1-μm sections were stained with 1% toluidine blue saturated with Li_2CO_3 for high resolution light microscopy with a Nikon Biophot or Optiphot microscope. Thin sections were collected on naked copper mesh grids and stained with uranyl acetate and lead citrate before being observed with a JEOL JEM-100CX II transmission electron microscope at the Department of Anatomy and Cell Biology, SUNY, Health Science Center, Syracuse.

Morphometry

Quantitative measurements from light and electron micrographs were used to determine the entrance aperture of the eye, the number of ommatidia, the volume density of the rhabdom, and the area of microvillar membrane per unit volume of rhabdom. The total surface area of the retina was determined from 1-μm sections. The average area occupied by an individual ommatidium was then measured and the number of ommatidia calculated from the total retinal area. The volume density of the rhabdom was determined from high magnification light micrographs using point-counting stereology. The average dimensions of the microvilli were determined from electron micrographs and the surface area of membrane per unit volume of microvillar array was then calculated assuming hexagonal close packing of the microvilli.

Results

Habitat

The small orange shrimp aggregates in swarms of hundreds, probably thousands of individuals on the sides of black smoker chimneys at the Beehive Mound of the Snake Pit Site at a depth of 3500 meters. Figure 2A shows a group of orange *Rimicaris* sp. above a mass of grey *R. exoculata.* Swarms of *Rimicaris* sp. consistently occupy particular regions of the environment and reestablish their position after they are disturbed by *Alvin's* collection activities. Temperature measurements suggest that *Rimicaris* sp. prefers an average ambient temperature of about 10°C compared to a higher average ambient temperature of 28°C preferred by *R. exoculata* (C. L. Van Dover, pers. comm.). Compared to *R. exoculata, Rimicaris* sp. individuals move less actively within their swarm and are only very rarely seen swimming singly in the water column.

Gross anatomy

Figure 2B shows a frame from a dive videotape of a group of *Rimicaris* sp. The bright white dorsal eye is clearly visible on each animal. This species lacks *R. exoculata's* characteristic bulged gill coverings and is more cylindrical. Figure 2C shows a picture of a single live animal perched on a piece of sulfide chimney wall in an aquarium on board the R/V *Atlantis II* several days after it was collected. In a living animal, the dorsal eye appears as a distinctive white structure with two wings beneath the transparent colorless cornea. The bilaterally symmetric pair of rounded wings is joined anteriorly across the midline by a thin cylindrical strand. Subsequent dissection and sectioning revealed that each of the wings has a ventral extension along its lateral edge that increases the size of the retina. Unlike those of shallow-water shrimp, the eyes are not stalked, but lie under the anterior and dorsal carapace. There are no compound eyes in *Rimicaris* sp.

Stereomicroscopic examination of a wing of the dorsal eye through the pellucid cornea of a living animal reveals a roughly polygonal, hexagonal honeycomb with pale pinkish-purple transparent ommatidia separated by white partitions. The partitions have ruffles along the top edge next to the cornea. Away from the cornea, the partitions close off each compartment with a textured surface that resembles the bottom of a lobed gelatin mold. Where the lobes meet in the center there is a ring of tiny holes formed by the axons from the individual photoreceptors passing through the bottom of each compartment in the honeycomb. Counting either the lobes or the number of holes in the ring shows that most ommatidia have seven photoreceptors, although a few have six, or even five, photoreceptors. Dissecting the eye of a live animal reveals that the inner surface of the white retinal matrix is lined with scattered aggregations of brownish-purple pigment. The colorless photoreceptor axons leaving the inner surface of the retinal wing on each side funnel ventrally toward the brain like the bristles of a bilateral pair of brooms. Each broom connects to one of a pair of dorsal visual neuropils that sit laterally on the brain.

Structure

Figure 3A shows a 1-μm section stained with toluidine blue cut through a wing of the dorsal eye tangential to the cornea where it overlies the center of the wing. The circumference of cornea and corneal epidermis surrounds darkly stained, light-sensitive rhabdom. Figure 3B shows a higher magnification view of the central region of Figure 3A. The rhabdomeral segments (R-segments) of the photoreceptors of each ommatidium cannot be individually distinguished as they contribute to the large volume of rhabdom. Thin partitions of white diffusing cells separate the ommatidia. Figure 3C shows a higher magnification view of the cornea, corneal epidermis, and underlying rhabdom. The cornea is smooth without evidence of any of the dioptric apparatus typical of an invertebrate compound eye. The corneal epidermis is separated from the layer of photoreceptors by a thin space filled with blood that occasionally contains hemocytes (Hose *et al.,* 1990). There are no cells that correspond to cone cells in shallow-water shrimp.

The average cross-sectional area of the R-segments of an individual ommatidium (*e.g.,* from Fig. 2B) and the total surface area of one half of the bilateral eye were used, in one animal, to estimate that each half of the eye contains between 3200 and 3400 ommatidia. In calculating the surface area of the eye, the ventral extensions on the lateral edges of the wings of the retina were included. From measurements of two 1-μm sections through the arrayed R-segments, we found that the rhabdom occupies between 80% and 85% of the total volume of the zone formed by the R-segments.

Figure 4 shows a section through the retina perpendicular to the corneal surface. The prominent blocky R-segments are separated and underlain by a layer of white

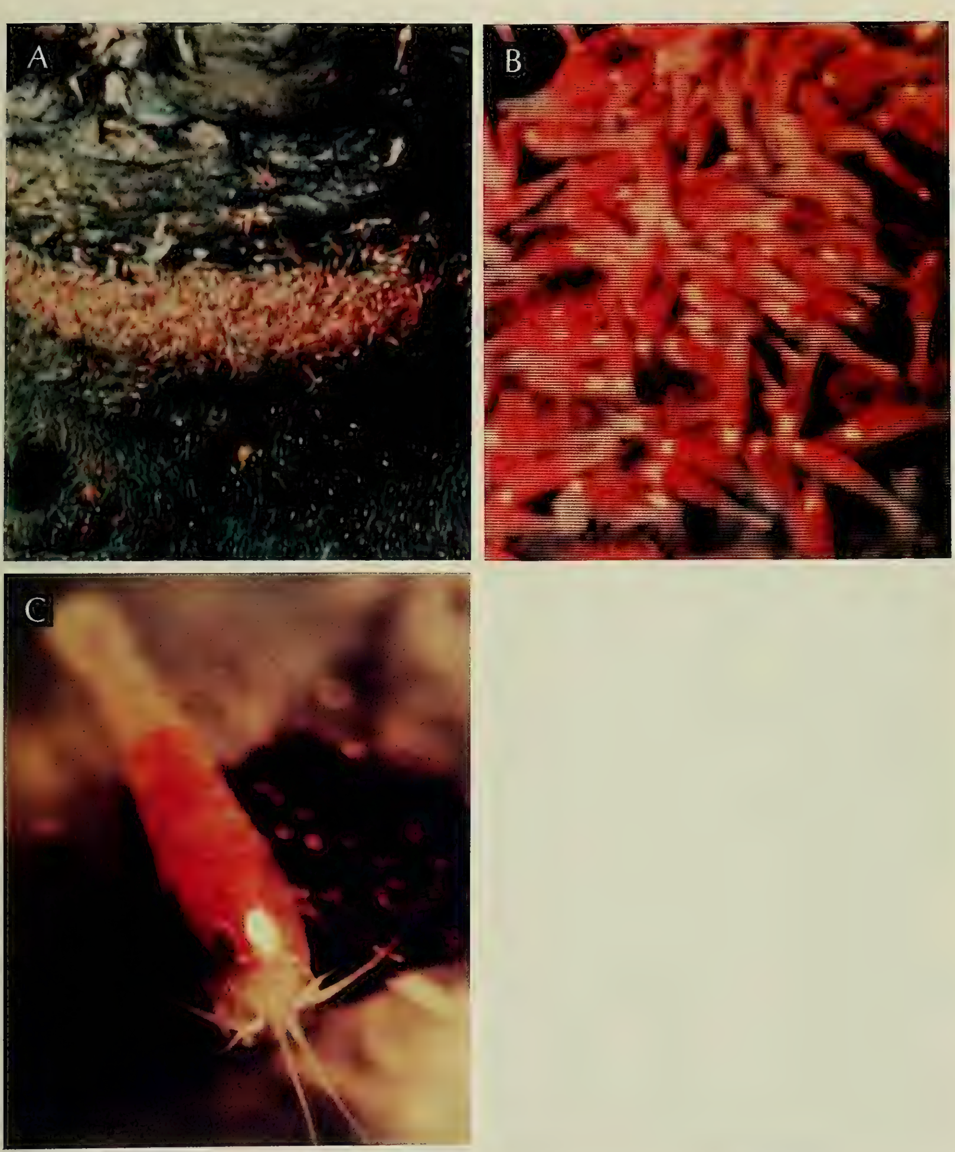

Figure 2. Views of *Rimicaris* sp. from the Snake Pit hydrothermal vent field. (A) An oblong swarm of animals at the Beehive Mound. The grey animals at the bottom are the species *Rimicaris exoculata.* Photograph from the sponson camera on DSV *Alvin.* (B) Video frame of *Rimicaris* sp. from the swarm in (A). The bright white structure on each animal is the dorsal eye. (C) Photograph of a single *Rimicaris* sp. sitting on a piece of vent chimney sulfide in an aquarium on board the R/V *Atlantis II.* Some individual animals survived at the surface for up to 21 days. The animals shown are about 3 cm in length.

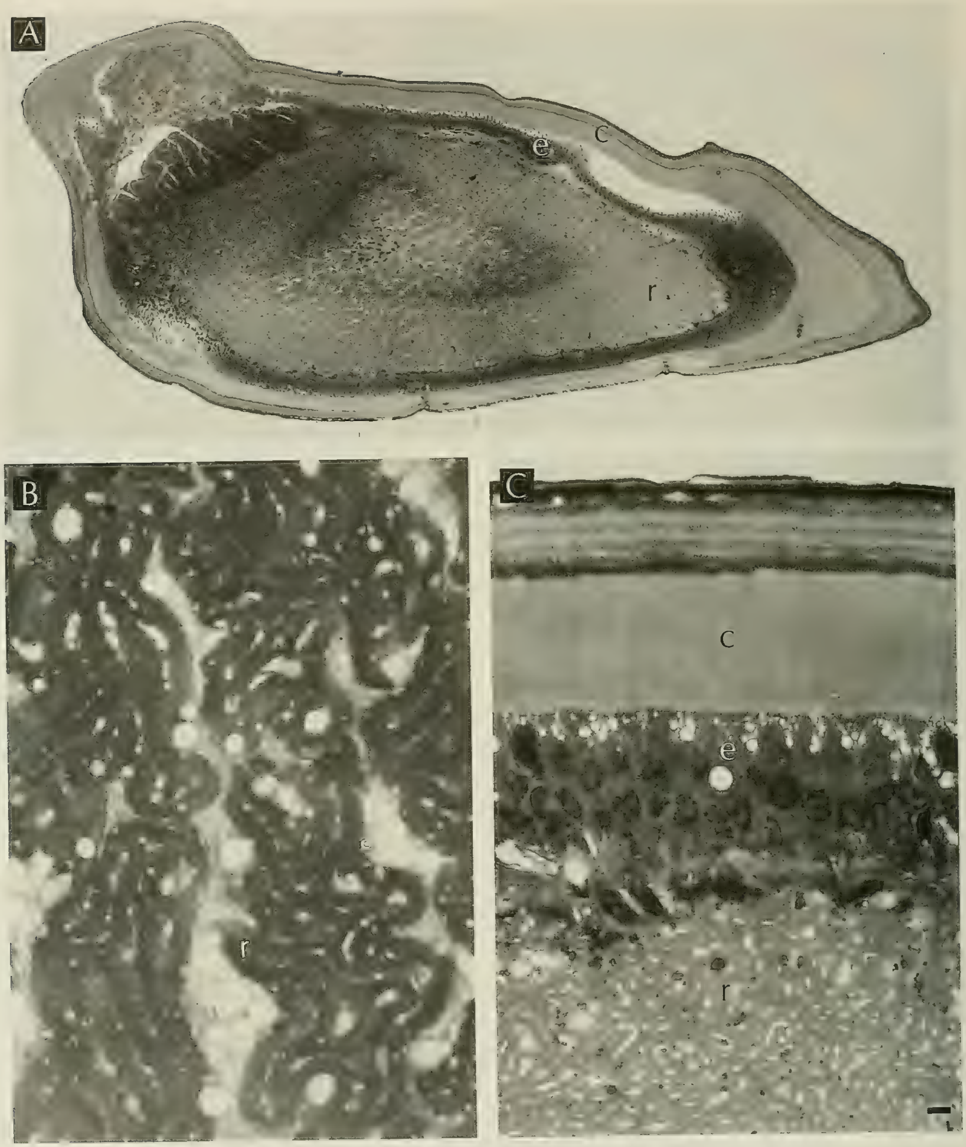

Figure 3. Light microscopy of 1-μm sections of the dorsal eye stained with toluidine blue. (A) Low magnification micrograph of a dorsal tangential section through one wing. The massed rhabdomeral segments (r) of the photoreceptors form a continuous sheet of photosensitive membrane surrounded by corneal epidermis (e) and cornea (c). The plane of section is tilted slightly from horizontal; anterior is to the left and medial toward the top. (B) Higher magnification micrograph of a more heavily stained section cut parallel to (A) through the rhabdomeral segments of the photoreceptors. The darkly stained regions are the microvillar arrays of the rhabdom separated by thin partitions of white diffusing cells. (C) An oblique section through the cornea (c), corneal epidermis (e), and rhabdomeral segments of the photoreceptors (r). The boundary between the corneal epidermis and the photoreceptors contains flattened hemocytes. The scale bar in (C) represents 63 μm in (A), 4.0 μm in (B), and 7.1 μm in (C).

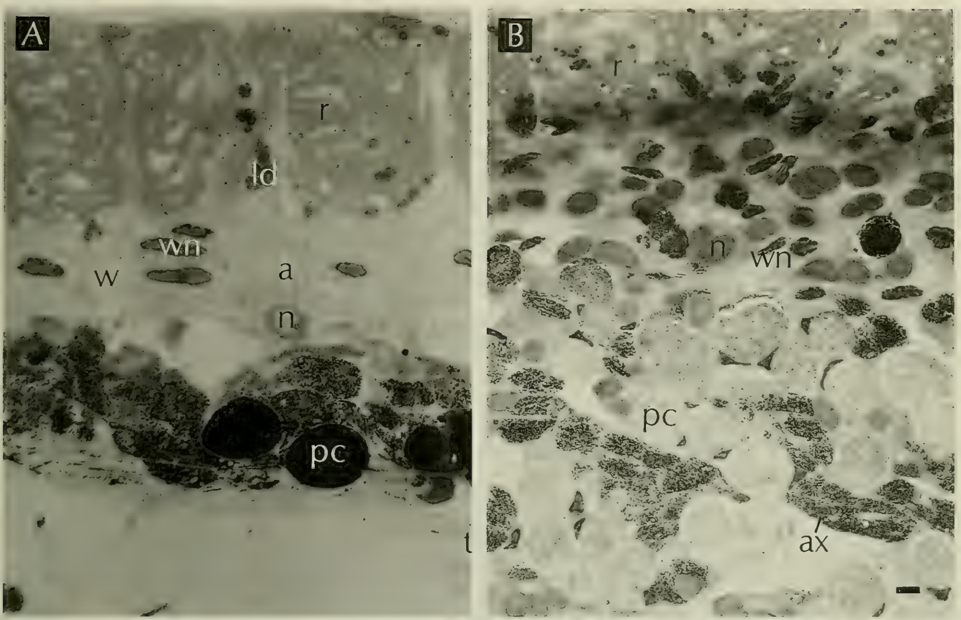

Figure 4. Light microscopy of 1-μm sections through the retina stained with toluidine blue. (A) Section perpendicular to the dorsal surface of the eye. The massed rhabdomeral segments (r) of the photoreceptors contain clumps of lipid droplets (ld) and are separated and underlain by white diffusing cells (w). Thin lucent cylindrical arhabdomeral segments (a) penetrate the thick layer of white diffusing cells. Toward the inner surface of the white diffusing cell layer, the arhabdomeral segment swells to surround the photoreceptor nucleus (n) before continuing toward the brain as a photoreceptor axon. Lobulated nuclei (wn) dot the layer of white diffusing cells. Ovoid pigment cells (pc) filled with screening pigment granules form an intermittent layer along the inside of the retina. (B) Section nearly perpendicular to the plane of (A) showing the inner surface of the layer of rhabdomeral segments (r), sequentially underlain by the the nuclei of white diffusing cells (wn) and photoreceptors (n), and pigment cells (pc) and screening pigment granules in the photoreceptor axons (ax). The scale bar in (B) represents 8.0 μm in (A) and 9.7 μm in (B).

diffusing cells. The ventral regions of the R-segment cytoplasm consistently contain small clusters of lipid droplets. The scattered nuclei of the white diffusing cells are angular in contrast with the more rounded photoreceptor nuclei that form a layer along the inner edge of the white diffusing cells. Below the zone of photoreceptor nuclei is a band of screening pigment, both in pigment cells and in the axons of photoreceptors. The pigment cells are uniformly ovoid with a range of pigment concentrations from very light grey to opaque black in toluidine blue-stained plastic sections. The arhabdomeral segment (A-segment) of the photoreceptor (analogous to the inner segment of a vertebrate photoreceptor) consists of a thin cylindrical strand that connects the base of the R-segment with the nucleus and, after swelling around the nucleus, continues as the axon. Granules of screening pigment in the photoreceptor axons are uniformly black and form elongated and striated arrays depending upon the plane of section.

Ultrastructure

Figure 5 shows the microvillar organization of the rhabdom. Most of the R-segment is occupied by geometrically ordered arrays of microvilli, and only a small volume is dedicated to the R-segment cytoplasm. The cylindrical microvilli are roughly hexagonally packed (Fig. 5 inset) and average only 0.09 μm in diameter with an average length of about 2.35 μm. The array provides about 40.3 μm^2 of photosensitive membrane per 1 μm^3 of rhabdom. The R-segment cytoplasm contains smooth endoplasmic reticulum, scattered mitochondria (Fig. 5), and clusters of lipid droplets along the base of the R-segment (Fig. 6A). The A-segment cytoplasm is relatively devoid of organelles except for the nucleus and a thin surrounding perikaryon (Fig. 6B,C) that contains mitochondria, but little else. Screening pigment granules are scattered in the cytoplasm of the axon below the photoreceptor nucleus.

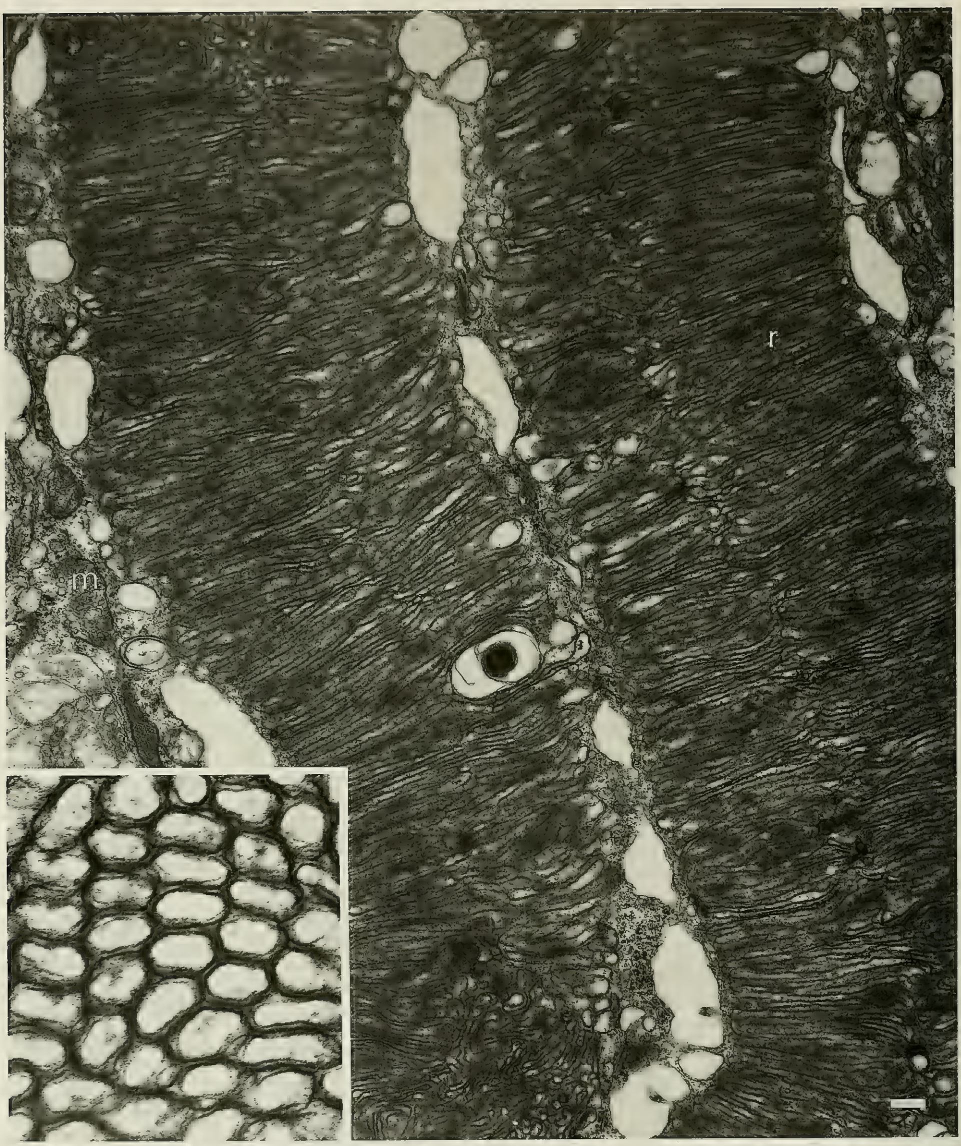

Figure 5. Electron micrograph of the rhabdom (r). The microvillar array is well organized. The cytoplasm has principally vacuoles and mitochondria (m). The inset shows a higher magnification micrograph of the microvilli in cross section. The scale bar represents 0.66 μm in the main micrograph and 0.09 μm in the inset.

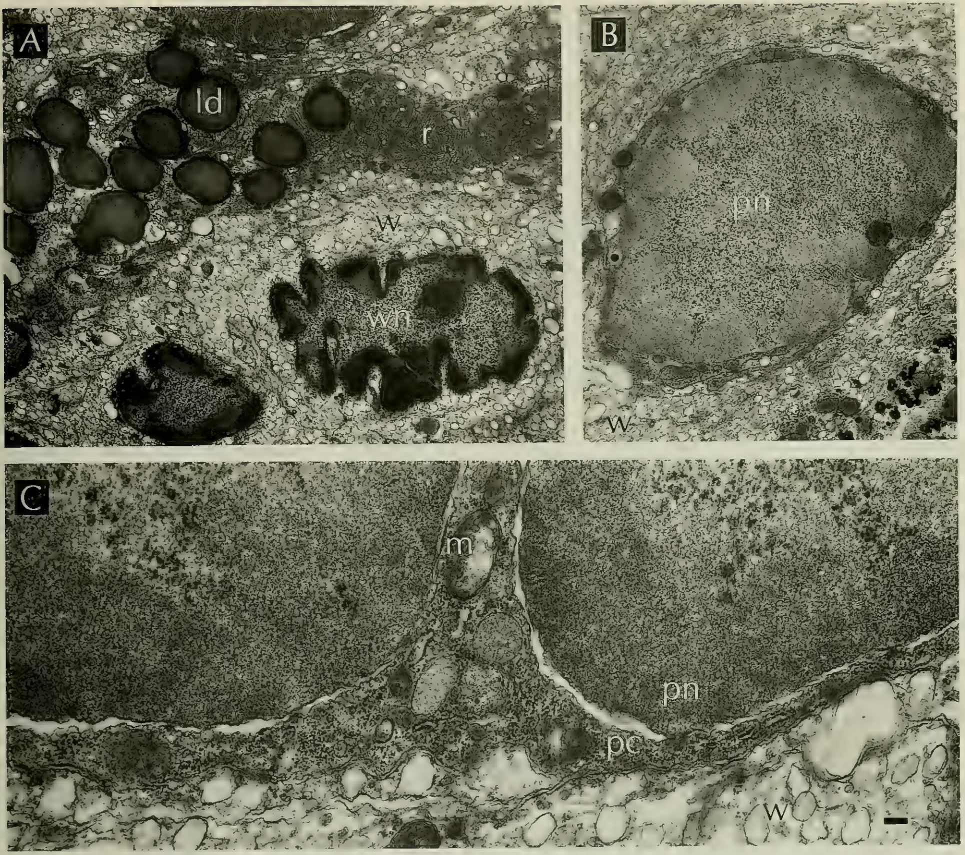

Figure 6. Electron micrographs of the retina. (A) Lobulated nucleus (wn) surrounded by the vesicle-laden cytoplasm of white diffusing cells (w). The adjacent photoreceptor rhabdomeral segment contains an aggregate of lipid droplets (ld) and rhabdom (r). (B) Photoreceptor nucleus (pn) is surrounded by a very thin band of cytoplasm and processes of white diffusing cells. The screening pigment granules at lower right are in the axon of another photoreceptor. (C) Higher magnification micrograph of the limited photoreceptor cytoplasm (pc) surrounding the nucleus (pn). Disrupted mitochondria (m) are present. The vesicles of the white diffusing cells (w) are adjacent to the photoreceptor cytoplasm. The scale bar in (C) represents 0.90 μm in (A), 0.76 μm in (B), and 0.19 μm in (C).

No structures typically associated with membrane shedding, such as lamellar bodies or multivesicular bodies, are found anywhere in the photoreceptor cytoplasm, nor are they found in the surrounding white diffusing cells.

The white diffusing cells have a complex shape with many long, thin branches. Sections show many small profiles with round vesicles in lucent cytoplasm. The white cell nucleus is prominently lobulated with distinct agglomerations of chromatin (Fig. 6A). Screening pigment cells are elliptical with cytoplasm replete with pigment granules and lobulated nuclei with aggregated chromatin.

Summary

Figure 7 is a summary drawing of the anatomy of the large dorsal eye of the bresiliid shrimp, *Rimicaris* sp. The paired wings are connected anteriorly by a thin strand that crosses the midline (Fig. 7B). The wings themselves have both a dorsal and lateral portion (Fig. 7C). The retina underlies a smooth, transparent cornea devoid of any dioptrics. The massive array of photosensitive membrane in the mosaic of photoreceptor R-segments sits on top of a layer of white diffusing cells that presumably reflect any

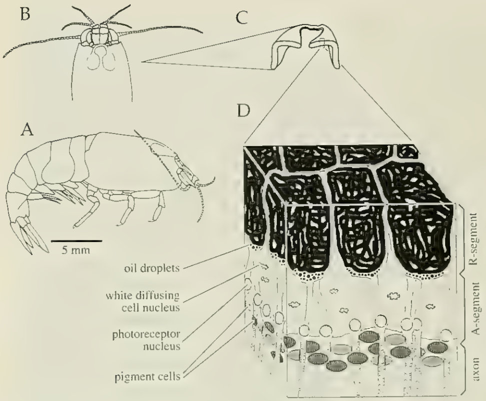

Figure 7. Summary figure. (A) Lateral view of *Rimicaris* sp. showing the position of its dorsal eye. (B) Dorsal view of the anterior portion of *Rimicaris* sp. showing both halves of the bilateral eye and its anterior portion across the midline. (C) A cutaway sketch of the retina showing the ventrally plunging lateral portions. (D) Schematic diagram of the organization of the retina showing the major cell types.

light that is not absorbed in the first pass through the rhabdom back into the rhabdom (Fig. 7D). Beneath this matte reflecting layer is a zone of screening pigment. Thin strands of photoreceptor A-segments penetrate the white diffusing cell layer to swell around the photoreceptor nuclei and continue toward the brain as photoreceptor axons.

Discussion

Comparison of Rimicaris *sp. with* R. exoculata

Rimicaris sp. and *R. exoculata* differ in several ways. The first species is bright reddish orange, and the second is ivory when freshly molted, turning gray and then black as sulfide particles build up in the gills, and finally brown as the sulfide particles oxidize. *Rimicaris* sp. lacks the lateral enlargement of the gill coverings so characteristic of *R. exoculata,* and the difference in shape becomes very clear when same-sized individuals of the two species are placed side by side. The largest *Rimicaris* sp. individuals we have observed are about 3 cm in length, but individuals of *R. exoculata* are commonly 6 cm long or longer. Finally, *Rimicaris* sp. clusters in regions with ambient temperatures of about 10°C, sits relatively quietly in these clusters, and is rarely seen swimming in the water column. In contrast, *R. exoculata* prefers regions with warmer ambient temperatures of 28° to 30°C, is more active within its clusters, and is commonly seen swimming in the water column and exploring areas remote from the clusters.

Comparison of the primary visual organs shows that they have a common basic design, but differ in details. The position of the eye is similar in the two species, but the wings of *Rimicaris* sp. are nearly round whereas those of *R. exoculata* are elongated. Both eyes are fused across the midline anteriorly with slight ventral projections of the anterior margins; however, *Rimicaris* sp. has corneal bulges in the expected positions for compound eyes, whereas *R. exoculata* lacks any external remnant of anterior compound eye structures. The number and hexagonal packing of the ommatidia are similar, but the R-segments of each ommatidium of *R. exoculata* are elongated while those of *Rimicaris* sp. are nearly equant. The microvilli in the rhabdom of *Rimicaris* sp. are markedly smaller in diameter than those of *R. exoculata* (0.09 μm *versus* 0.15 μm). This leads to a greater concentration of photosensitive membrane in the rhabdom (40.3 $\mu m^2/\mu m^3$ *versus* 24.18 $\mu m^2/\mu m^3$) which may compensate for the smaller overall size of the *Rimicaris* sp. eye (10 mm^2 *versus* 27 mm^2). The clusters of lipid droplets present in the cytoplasm of the *Rimicaris* sp. photoreceptors are entirely absent from the photoreceptors of *R. exoculata. Rimicaris* sp. lacks any retinal cell type that could correspond to the cone cells that form the dioptrics of shallow-water shrimp, whereas the corneal epidermis of *R. exoculata* includes a minority cell type that appears to be an underdeveloped cone cell. The adaptation of the white diffusing cells, the attenuation of the A-segment, and the migration of the pigment granules in the photoreceptors and the pigment cells to the inner margin of the retina are shared features of the two species.

We conclude that *Rimicaris exoculata* and *Rimicaris* sp. are closely related, but distinct, species that have adapted for life in an unusual, low-light environment with similar modifications to their eyes. Both have enlarged, dorsal eyes that are similar in overall plan, but different in detail.

Comparison of Rimicaris *sp. with* Palaemonetes

We suggest that *Rimicaris* sp. is an evolutionary adaptation of a species of caridean shrimp that migrated from the surface at some time in the past, possibly in the last 5000 to 10,000 years—the typical lifetime of a hydrothermal vent field. This idea arises from the observation that three of the four vent fields on the Mid-Atlantic Ridge have different majority shrimp populations and seem not to share many species in common. It may, therefore, be interesting to compare the structure of the *Rimicaris* sp. eye with that of shallow-water species such as *Palaemonetes pugio* (Itaya, 1976; Doughtie and Rao, 1984) or *P. vulgaris* from which it may have evolved, perhaps through many intermediates.

We estimate that each wing of the eye of *Rimicaris* sp. contains 3200 to 3400 ommatidia. This compares favorably with the 3600 ± 300 ommatidia reported for the eye of *P. pugio* or the 2800 ommatidia we estimated for the

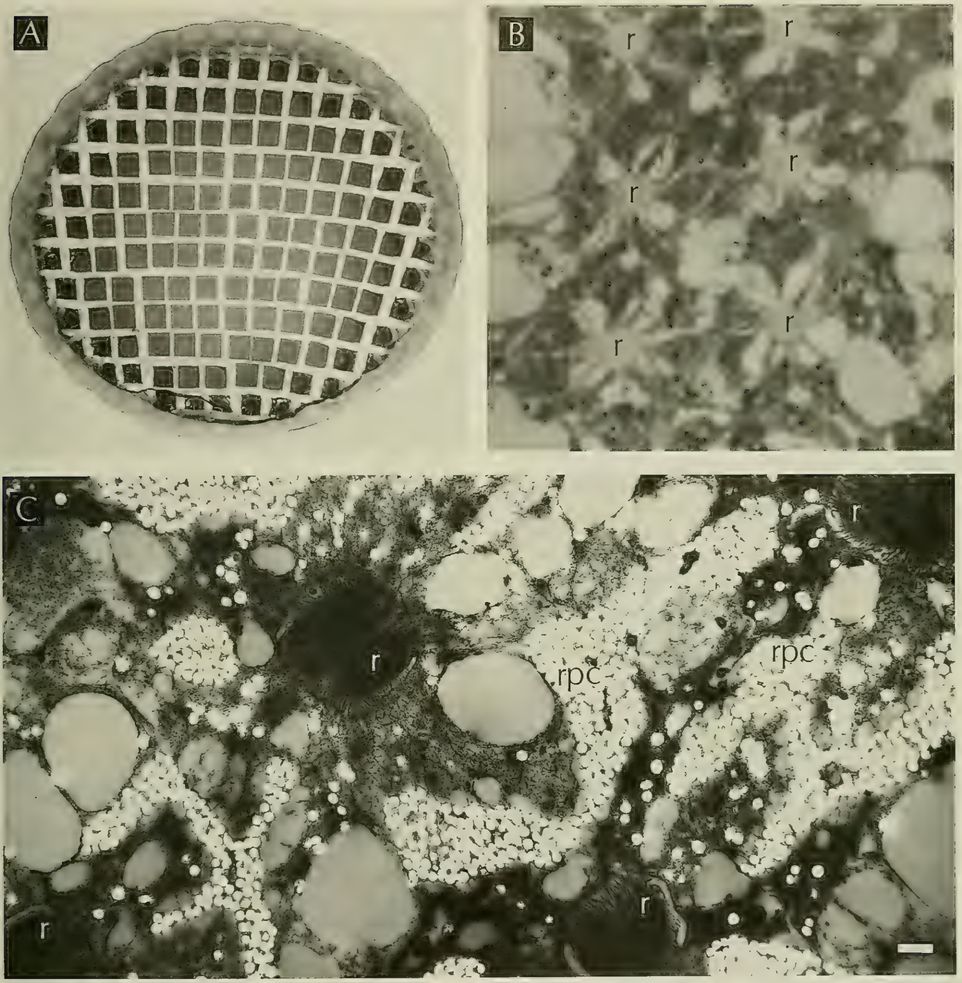

Figure 8. Micrographs of the eye of the grass shrimp, *Palaemonetes vulgaris.* (A) One-micrometer plastic section through the dioptric apparatus. The four cone cells that form each crystalline tract are visible in the center of the array. (B) Higher magnification light micrograph of a 1-μm section through the photoreceptors. Each rhabdom (r) consists of a cylinder of tightly packed microvilli that occupy relatively little of the retinal volume. (C) Electron micrograph showing the rhabdoms (r) with interspersed reflecting pigment cells (rpc). The scale bar in (C) represents 68 μm in (A), 4.6 μm in (B), and 1.7 μm in (C).

eye of *P. vulgaris.* The overall entrance aperture for one wing is nearly planar and about 5 mm^2 in *Rimicaris* sp. and spherical and about 5.6 mm^2 in *P. vulgaris.* When the retina is considered as a slab of tissue, the rhabdom comprises about 10% of the volume at the level of the R-segments in *P. pugio* and 13% in *P. vulgaris.* The corresponding volume density of rhabdom in *Rimicaris* sp. ranges from 80% to 85%.

The geometrically arranged dioptric apparatus of *Palaemonetes* (Figs. 8A, and 9) has been replaced in *Rimicaris* sp. by a smooth cornea (Fig. 3A, C) lined by a corneal epidermis without the cone cells that form the quadri-

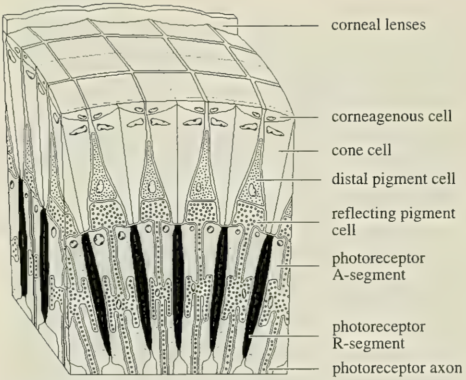

Figure 9. Schematic diagram of the organization of the retina of the compound eye of *Palaemonetes* showing the major cell types. This drawing is based on examination of *Palaemonetes vulgaris* in this study and published results for *Palaemonetes pugio* (Doughtie and Rao, 1984—figure 1) and does not include the effects of light and darkness or time of day on the structure of the retina.

partite dioptrics in *Palaemonetes.* The distal pigment cells in *Palaemonetes* that contain opaque screening pigment granules are probably represented by the ellipsoidal pigment cells along the inner surface of the retina in *Rimicaris* sp. The reflecting pigment cells, which in *Palaemonetes* are filled with vesicles, are probably represented by the elaborated white diffusing cells in *Rimicaris* sp. The screening pigment granules in the photoreceptors that migrate between the axons and the R-segment in response to light and darkness in *Palaemonetes* are reduced in number in *Rimicaris* sp. and seem to be restricted to the axons and the A-segment below the nucleus. The geometrical, interleaved arrangement of microvilli from the rhabdomeres of individual photoreceptors in *Palaemonetes* (Fig. 8B, C) is replaced by a much larger, but less geometrical rhabdom in *Rimicaris* sp. Table I compares the classes of retinal cells of *Palaemonetes* and *Rimicaris* sp.

The suggested evolution of the stalked eyes of *Palaemonetes* into the dorsal eye of *Rimicaris* sp. can be summarized as follows (compare Figs. 7 and 9). The dioptric apparatus is gone. Although the number of ommatidia is unchanged, each is larger so that the volume of the eye is increased. The sensitivity of the retina is likely further enhanced by a sevenfold to eightfold increase in the volume density of photosensitive membrane in the rhabdomeres of the R-segments. Screening pigment granules have been removed from the light path between the cornea and the rhabdom and have been decreased in number and prominence. The array of white diffusing cells with high albedo that increases the quantum catch by back

Table I

Comparison of retinal cell types: Palaemonetes *and* Rimicaris *sp.*

Palaemonetes[†] Cell Type	Description	*Rimicaris* sp. Cell Type	Description
Corneagenous cells	Modest continuous layer between corneal lenses and cone cells.	Corneal epidermis cells	Prominent continuous layer underlying smooth cornea.
Cone cells	Four cone cells form a quadripartite crystalline tract for each ommatidium. Occupy half the thickness of the retina.	None	Only hemocytes occupy the space between the corneal epidermis and the photoreceptor R-segments.
Distal pigment cells	Fill space between crystalline tracts distal to the photoreceptors. Migrate with light and darkness. Give eye black color.	Pigment cells	Ovoid cells proximal to photoreceptors and white diffusing cells. Not in light path.
Reflecting pigment cells	Partition photoreceptor clusters. Adjacent to rhabdom in dark adapted retina. Elongated in light adapted retina.	White diffusing cells	Partition photoreceptor clusters and form thick diffusing layer proximal to the photoreceptor R-segments. Give eye white color.
Photoreceptors		Photoreceptors	
R-Segment	Cylindrical rhabdom contains microvilli from 8 retinular cells. Makes up 10% of proximal retina by volume.	R-Segment	Extensively infolded rhabdom makes up 80% of distal retina by volume. Lipid droplets common proximally.
A-Segment	Forms major portion of photoreceptor. Nucleus at level of distal rhabdom. Screening pigment granules adjacent to rhabdom and in axon. Lipid droplets, rough endoplasmic reticulum, and mitochondria are common.	A-Segment	Minor portion of photoreceptor consisting of thin cylindrical connection with R-segment, nucleus, and axon. Few organelles in cytoplasm. Nucleus proximal to R-segment and all screening pigment granules proximal to nucleus.

[†] From Doughtie and Rao (1984).

scattering light into the photoreceptors has been markedly increased compared to the reflecting pigment cells in *Palaemonetes.* Taken together, these adaptations represent a significant attempt to maximize quantum catch at the cost of a loss of imaging capabilities. The eye is no longer an organ for pattern vision, but has become a very sensitive light detector. These structural adaptations are consistent with those observed in three species of deep-water mysid crustaceans by Elofsson and Hallberg (1977).

The structural changes observed in the retina of *Rimicaris* sp. might be expected in an animal that is adapting to a dim-light environment (*e.g.,* Lythgoe, 1979) and are consistent with the changes we have observed in other hydrothermal vent shrimp. In general, an animal's eye continues to enlarge as the environment becomes dimmer and dimmer until the "quit point" is reached where exploitation of light in the environment becomes impossible (Lythgoe, 1979). In animals that adapt to the darkness beyond the quit point, the eye shrinks or disappears altogether. The eyes of the bresiliid shrimp that live on the sides of black smoker chimneys at sites along the Mid-Atlantic Ridge have all enlarged (Chamberlain *et al.,* 1994a,b; O'Neill *et al.,* 1995); however the eye of the predator *Alvinocaris markensis,* which lives at the base of these chimneys, seems to be in the process of evolving past the quit point (Wharton *et al.,* unpub. data).

Adaptation for vision at hydrothermal vents

Does any light reach this hydrothermal vent environment from the surface? Clarke and Denton (1962) estimated that in the very clearest seawater, fish ought to be able to detect daylight to a depth of 1000 m. Clarke and Kelly (1964) measured light penetration in the Indian Ocean and concluded that the greatest depth for vision might vary between 700 m and 1300 m. This is the very range of depths that separates the twilight zone inhabited by mesopelagic fauna with well-developed eyes from the dark zone inhabited by bathypelagic fauna that usually have regressed eyes. Foxton (1970) reported that a typical faunal break for decapod crustaceans occurred between 650 m and 700 m. Kampa (1970) reported that at this depth in the North Atlantic, the light energy from the surface is about 10^{-6} μW cm^{-2} nm^{-1} at 480 nm. In any case, at a depth of 3500 m there is negligible light penetrating from the surface.

During our dive series, one of us (E. D. H.) tested whether a profoundly dark-adapted human observer could detect any light in the ambient environment at the TAG Site and saw nothing. This observation was repeated at the Snake Pit Site (Carolyn Eberhard, pers. comm.) with the same negative results. Since bioluminescence should be visible to human observers, this suggests that there is no significant bioluminescence, or light from any other source bright enough to be visible to human observers, under the conditions of a visit with DSV *Alvin.* We are left, then, with the light apparently emitted at the top of the black smoker chimneys (Van Dover *et al.,* 1994) as the most likely possibility. This light might well be black-body radiation, but other mechanisms that could produce such light have recently been suggested (Van Dover *et al.,* 1994). The intensity, spectrum, and distribution of light emitted at hydrothermal vents needs to be investigated experimentally before firm conclusions can be reached about the underlying mechanisms of light production. The spectral sensitivity of the dorsal eye of *R. exoculata* seems to be maximum around 500 nm whether determined from scans of retinal extracts (Van Dover *et al.,* 1989) or electroretinographic responses to lights of varying wavelength (Johnson *et al.,* 1995).

The masses of *Rimicaris* sp. which swarm around the sides of the chimneys would, then, likely be in the shadow that the chimney lip would cast. The dorsal position of the eye would be appropriate, for example, to signal that an animal had moved away from the mass and out of the shadow into the light that is coming from above. This could provide a local navigational cue to guide an animal back to the mass if it swam far enough away to emerge into the light. Likewise, as animals crept higher and higher on the sides on the chimney toward the lethally hot water of the emerging jets, the shadow would become narrower and they would more easily emerge into brighter light which could warn them of the potential danger of being swept into the hot jet.

It is likely that hydrothermal vent communities lack daily environmental cues. Do vent organisms continue to show the daily rhythms in physiology and behavior typical of their shallow-water relatives? We observed no organelles typical of a cyclic shedding and renewal of the photosensitive rhabdom in the photoreceptors or accessory retinal cells of *Rimicaris* sp. Except at the corneal surface, the R-segments are completely sheathed in white diffusing cells whose cytoplasm is filled with diffusing vesicles and little else. The corneal epidermis is separated from the tips of the R-segments by a blood space. Thus it seems unlikely that a mechanism of extracellular rhabdom shedding is employed. Within the photoreceptors, the volume density of rhabdom is so high (80%–85%) that there is little space available for rhabdom shedding to occur compared to a species such as *Limulus polyphemus* where the rhabdom occupies about 2% of the retina overall or 35% to 40% of the photoreceptor R-segment. However, the high volume density itself is not conclusive evidence for the absence of rhabdom shedding since squid (Tsukita *et al.,* 1988), which sheds its rhabdom, has a volume density of rhabdom in its photoreceptors (75%) that is comparable to *Rimicaris* sp., but the rest of the squid photoreceptor is not attenuated.

The small size of the A-segment in *Rimicaris* sp., especially compared to the very large amount of microvillar membrane in the R-segment, makes it unlikely that significant cycling of photosensitive membrane can be occurring (Hornstein and Chamberlain, 1991; Chamberlain and Hornstein, 1993). This conclusion is reinforced by the restricted cytoplasm around the nucleus and the very limited amount of endoplasmic reticulum of any kind for protein synthesis and subsequent membrane assembly. Indeed, the large array of microvillar membrane in a mature photoreceptor with so little synthesis machinery in evidence raises questions about how the array is produced during the growth and development of the juvenile animal and suggests that the relative atrophy of the A-segment may be a feature only of the mature animal.

Acknowledgments

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Classification of Nerve Cells Dissociated From Tentacles of the Sea Anemone *Calliactis parasitica*

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Abstract. Nerve cells from tentacles of the sea anemone *Calliactis parasitica* were dissociated in 1000 units/ml of collagenase for scanning electron microscopic studies and in 0.125% elastase followed by 12 units/ml of ficin for cell counts using light microscopy. The studies revealed 33 distinguishable neuronal shapes, which were categorized as either sensory cells having an apical cilium or ganglion cells with or without a perikaryal cilium and further subdivided into unipolar, bipolar and multipolar neurons based on the number of processes that extended from the perikaryon. Unipolar sensory cells were characterized by an apical cilium adjacent to the perikaryon and a long, simple or terminally branched axon. Unipolar ganglion cells lacked an apical cilium. Bipolar sensory cells had a neck between the cilium and perikaryon and an oppositely directed axon. Bipolar ganglion cells had isopolar processes or asymmetrical processes, which were simple or complexly branched. One type of bipolar ganglion cell with isopolar processes had a perikaryal cilium. Multipolar sensory cells had a distinct neck between the perikaryon and the cilium and two or more simple or complexly branched processes extending from the triangularly shaped cell body. Multipolar ganglion cells had variously shaped perikarya from which extended three or more short or long processes that were simple or complexly branched. One type of tripolar ganglion cell had a perikaryal cilium. The different types of nerve cells were quantified, and statistical comparisons were made.

Introduction

The nervous system in tentacles associated with feeding in sea anemones includes ciliated sensory cells oriented perpendicularly to the surface and ganglion cells located above the basoepithelial musculature (Westfall, 1973b). Sensory cell axons either end near the mesoglea or bifurcate to join the nerve plexus formed by ganglion cells and their processes (Leghissa, 1965). Tracing the nerve cells to their synaptic sites on other cells by transmission electron microscopy (TEM) is difficult without understanding the overall morphology of the cell. Therefore, to supplement current TEM studies on synapses in tentacles of *Calliactis parasitica,* we initiated this cell dissociation study and were surprised to find a variety of sensory cells. A recent review of the microscopic anatomy of the anthozoan nervous system (Fautin and Mariscal, 1991) described only two morphological types of sensory cells in sea anemones and indicated a paucity of information on the ultrastructure of ganglion cells.

With the use of maceration techniques on the freshwater polyp *Hydra,* several nerve cell types have been dissociated and distinguished as either having an apical cilium and, therefore, being typical sensory cells, or having one to several processes that make them appear unipolar, bipolar, or multipolar in shape (Schneider, 1890; Hadži, 1909; David, 1973; Tardent and Weber, 1976; Epp and Tardent, 1978; Westfall and Epp, 1985; Yu *et al.,* 1986; Westfall and Rogers, 1990). Two attempts have been made to devise classification schemes for *Hydra* neurons (Tardent and Weber, 1976; Yu *et al.,* 1986). This study is the first attempt to create a classification scheme for sea anemone nerve cells.

New cell dissociation techniques using enzymes make it feasible to isolate nerve cells from the epithelium of sea anemone tentacles (Przysiezniak and Spencer, 1989). Such isolated neurons can be attached to a substrate suitable for fixing, dehydrating, and critical-point drying of the cells for subsequent examination by scanning electron microscopy (SEM). This method provides information on even minute neuronal structures, such as recessed cilia

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on sensory cells and short perikaryal cilia on ganglion cells, which are important in categorizing the cells into groups.

The purpose of this study was to categorize dissociated sea anemone neurons and develop a general classification scheme for these primitive sensory and ganglion cells. This may be of value in setting morphological criteria for comparing number and types of neurons in different species of coelenterates, regardless of neuronal size.

Materials and Methods

Cell dissociation for SEM

Small specimens of *Calliactis parasitica* (sent from Station Biologique, Roscoff, France) were maintained in an Instant Ocean and fed Invert-food (Ocean Nutrition, San Diego). We studied neurons in 14 tentacles from four animals. For the SEM studies, tentacles from two different specimens were extirpated with scissors and allowed to relax in artificial seawater for 10–15 min, then measured to the nearest millimeter, and transferred to silicon-coated test tubes containing divalent cation-free seawater (DCFSW). After 10–15 min, the tentacles were dissociated in 1000 units/ml of collagenase Type IA (Sigma Chemical Company, St. Louis, Missouri) following the method described by Przysiezniak and Spencer (1989). After 2.5–3 h at room temperature (RT), the cells were rinsed with DCFSW, some of which was pipetted off to leave a volume of 75–100 μl. The tissue was triturated immediately, using a silicon-coated Pasteur pipette, and 25 μl of the cell suspension was transferred to coverslips coated with Cell-Tak (Collaborative Biomedical Products, Bedford, Massachusetts). The cells were fixed by adding a drop of 2.5% glutaraldehyde in 0.05 *M* sodium cacodylate buffer, pH 7.4, osmolality 357 mmol/kg, and allowed to settle on the Cell-Tak-coated coverslips in a moisture chamber for 24 to 36 h at RT. Undissociated pieces of mesoglea were removed. The coverslip with attached cells was processed through increasing concentrations of ethanol and critical point dried in CO_2. A 10-mm-diameter copper grid with locator markings (Ted Pella, Inc., Redding, California) was attached to the coverslip to map the cells by phase contrast microscopy before they were coated with gold-palladium for SEM using an ETEC Autoscan.

Observations also were made by phase microscopy to check the rate of dissociation of the neurons and any potential degenerative changes with enzyme incubation, such as perikaryal swelling or segmentation and loss of processes. The loss of cells during settlement and processing was checked by observing a drop of cell suspension from the same preparation both before and after addition of fixative. These cells were retained as temporary preparations for comparison with fixed cells allowed to settle for 24–36 h on coverslips coated with Cell-Tak, and then dehydrated in ethanol and critical point dried. In addition, loss of cells during processing through increasing concentrations of ethanol was checked by observing the washings by phase microscopy and retaining the cells as temporary preparations. Light micrographs were taken, and camera lucida drawings were made of representatives of each identifiable cell type. The drawings were cut out and arranged into categories based on the number of processes arising from the perikaryon and the presence of an apical cilium. Neurons representing each category were measured with an ocular micrometer and mapped before they were metal coated for SEM. Many neurons subsequently located by SEM matched the camera lucida drawings. Others were lost during processing, coating, or searching by SEM. Some neurons were too long and slender to be effectively imaged by SEM or were surrounded by debris making them unsuitable for publication. However, some neurons and other cell types were retained in good condition after processing for SEM.

Cell counts

Nerve cell counts for statistical comparisons were made from a total of four tentacles, two each from two animals. The tentacles were placed in DCFSW as explained above and dissociated using a solution of 0.125% elastase (Sigma Chemical Company) followed by 12 units/ml of ficin (Sigma Chemical Company) in DCFSW at 35–37°C for 2 h each at RT. The cells were rinsed in DCFSW and transferred to Cell-Tak-coated coverslips as described above, then fixed in glutaraldehyde, dehydrated in ethanol, and critical point dried. A 10-mm SEM finder grid was attached to a coverslip of critical-point-dried cells, and then the total number of identifiable nerve cells was counted for quantitative analysis using phase contrast microscopy. Statistical comparisons were made using ANOVA followed by Fisher's LSD test.

Results

Classification scheme

Based on SEM of sensory cells (Figs. 1–11) and ganglion cells (Figs. 12–28) and a combination of camera lucida drawings and light micrographs, we could distinguish 33 types of sea anemone neurons, which were incorporated into a classification scheme (Fig. 29). Isolated nerve cells were categorized into unipolar, bipolar, and multipolar neurons as follows:

I. Apical perikaryon with single slender process = unipolar neuron (top row, Fig. 29)
    A. Cilium associated with apical perikaryon = unipolar sensory cell (Figs. 1–4)
    B. No cilium on apical perikaryon = unipolar ganglion cell (Figs. 12–14)
II. Perikaryon with asymmetrical or symmetrical oppositely directed processes = bipolar neuron (middle group, Fig. 29)

  - A. Cilium at end of short neck = bipolar sensory cell (Figs. 5–7)
  - B. Cilium on perikaryon = ciliated bipolar ganglion cell (Fig. 15)
  - C. No cilium on perikaryon = bipolar ganglion cell (Figs. 16–20)
- III. Perikaryon central to three or more processes = multipolar neuron (bottom group, Fig. 29)
  - A. Cilium at end of neck = multipolar sensory cell (Figs. 8–11)
  - B. Cilium on perikaryon = ciliated multipolar ganglion cell (see c on unnumbered tripolar neuron)
  - C. No cilium on perikaryon = multipolar ganglion cell (Figs. 21–28)

Unipolar sensory cells

Unipolar sensory cells: cilium or cilium-stereociliary complex adjacent to an apically located perikaryon with an oppositely directed axon. Based on the arrangement of the apical ciliary apparatus, unipolar sensory cells fall into four types: (1) a cilium, either projecting or recessed, surrounded by a ring of stereocilia forming an apical cone (Fig. 1); (2) a 3–7-μm-long cilium extending from a plain unadorned socket (Fig. 2); (3) a 7-μm-long cilium with a ring of short basal stereocilia (Fig. 3); and (4) a 4–5-μm-long cilium surrounded basally by a bonnet-like apical flare of cytoplasm (Fig. 4). All four types of unipolar sensory cells are elongate with a 2–7-μm-wide perikaryon and a slender, simple, beaded, or bushy axon with spatulate, tapered, lamellate, or branched terminals. The cells range in length from 20 to 67 μm (Table I).

Bipolar sensory cells

Bipolar sensory cells: short neck between the cilium and the perikaryon and an oppositely directed axon (Figs. 5–7). There are four morphological types: (1) a 3–7-μm-long cilium with a 1–2-μm-long neck, an 8–11-μm-long tapered perikaryal region, and a long unbranched axon with a simple terminal (Fig. 5); (2) a 7–8.3-μm-long cilium rising from the enlarged apex of a narrow 7–16-μm-long neck (Fig. 6); (3) a 4–7-μm-long cilium extending from a 1.5–7.7-μm-long neck and a short oppositely directed axon, which bifurcates into two long terminal processes (Fig. 7); and (4) a short, curved, neck-like process with a 5–6-μm-long apical cilium and a slender axon with a pair of terminal swellings having twig-like processes (not shown by SEM). The perikarya are 3.5–6.9 μm wide. The cells range in length from 47 to 290 μm (Table I).

Multipolar sensory cells

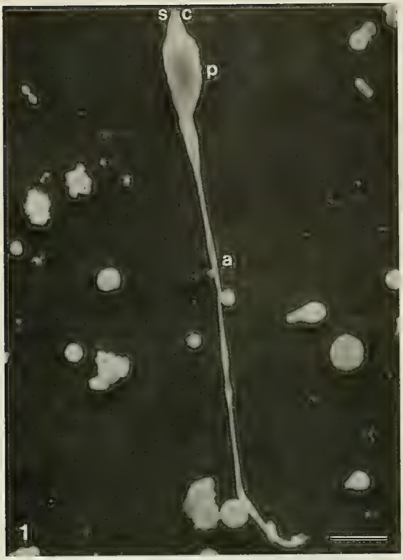
Multipolar sensory cells: cilium at the end of a short neck forming one of three or more processes that extend directly from the perikaryon (Figs. 8–11 and 29). Usually, the perikaryon appears triangular in shape and has oppositely directed basal processes. Four morphological variations include (1) a 3–5-μm-long cilium extending from a 3–5-μm-long neck (Fig. 8); (2) a 8–9-μm-long cil-

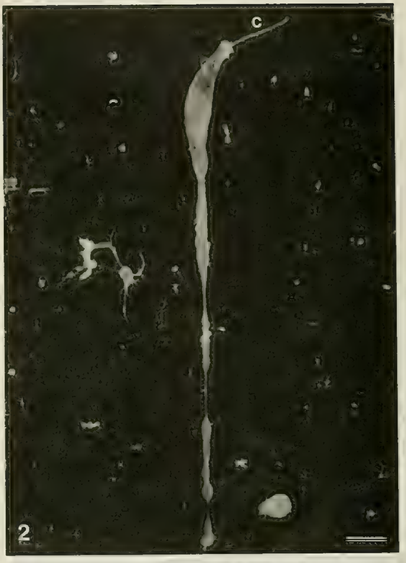
Table I

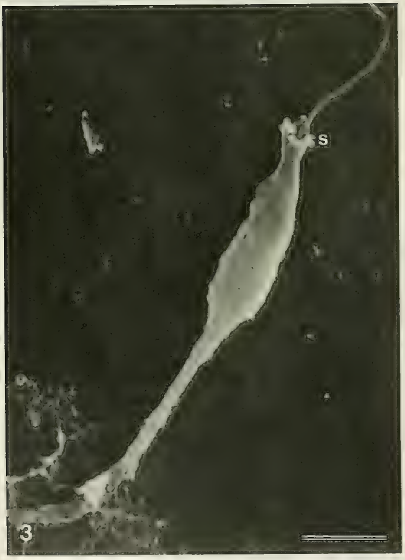
Type, length, and number of neurons counted in four sea anemone tentacles

Figure number	Type of cell	Cell length in μm	Number of cells counted*	% of total neurons counted	% of either sensory or ganglion cells
UNIPOLAR SENSORY CELLS:					
Fig. 1	With ciliary cone	20–48	492	7.91	29.2
Fig. 2	Single cilium	30–63	665	10.69	39.5
Fig. 3	Basal stereocilia	28–50	6	0.10	0.36
Fig. 4	Flared apex	35–67	4	0.06	0.24
BIPOLAR SENSORY CELLS:					
Fig. 5	Short neck	47–88	117	2.84	10.5
Fig. 6	Cilium from enlarged apex	55–290	148	2.38	8.8
Fig. 7	Narrow neck/axon bifurcation	50–84	4	0.06	0.2
Not shown	Curved neck	59–64	21	0.33	1.3
MULTIPOLAR SENSORY CELLS:					
Fig. 8	Triangular	11–49	62	1.0	3.7
Fig. 9	With long neck	18–94	24	0.38	1.4
Fig. 10	Complexly branched axons	93–107	27	0.43	1.6
Fig. 11	More than two axons	30–124	54	0.87	3.2
GANGLION CELLS:					
Fig. 12–14	Unipolar	31–71	516	8.29	11.4
Fig. 15–20	Bipolar	39–210	1312	21.07	28.9
Fig. 21–28	Multipolar	31–310	2705	43.50	59.7

* These numbers represent all nerve cells counted in a total of four tentacles from two different animals.







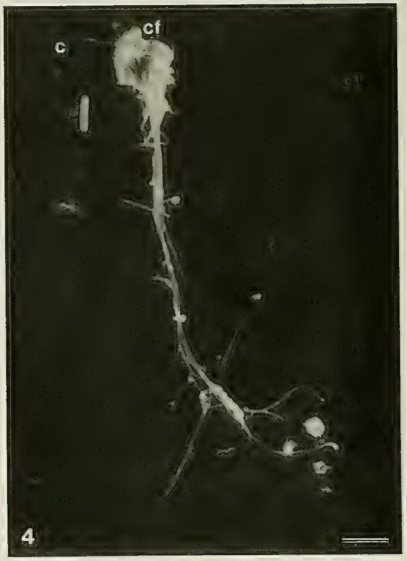
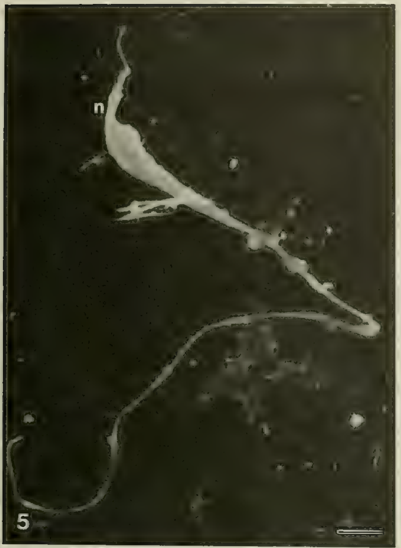


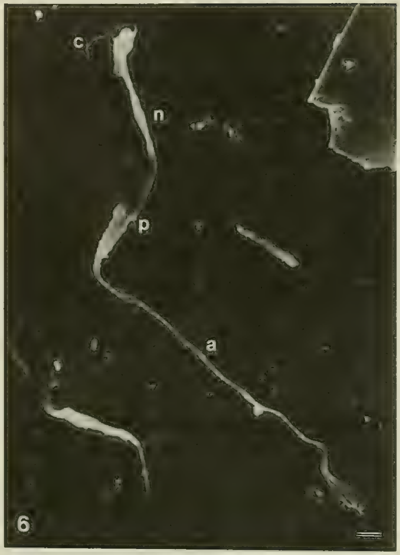
Figure 1. Scanning electron micrograph of unipolar sensory cell with cone of stereocilia (s) surrounding a recessed cilium (c) near the perikaryon (p) from which extends a slender simple axon (a). Scale bar = 3 μm.

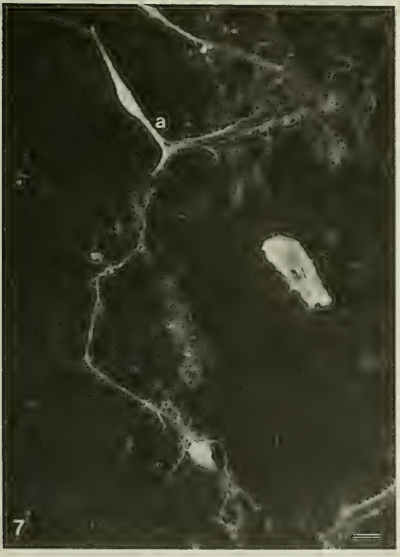
Figure 2. Unipolar sensory cell with projecting cilium (c), elongate perikaryon, and beaded axon. Scale bar = 3 μm.

Figure 3. Unipolar sensory cell with long cilium adorned by ring of basal stereocilia (s) adjacent to an elongate perikaryon with a simple axon. Scale bar = 3 μm.

Figure 4. Unipolar sensory cell with an apical cilium (c) surrounded basally by a cytoplasmic flare (cf) of the wide perikaryon from which extends an irregularly branched axon. Scale bar = 3 μm.







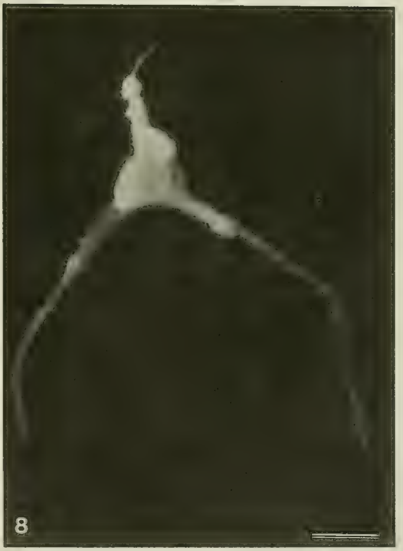


Figure 5. Bipolar sensory cell with an apical cilium arising from a short, apically tapered, neck (n) extending from a perikaryon with an oppositely directed, long, slender axon. Scale bar = 3 μm.

Figure 6. Bipolar sensory cell with an apical cilium (c) extending from a swelling at the apex of a neck (n) above the elongate perikaryon (p) and a long simple axon (a). Scale bar = 3 μm.

Figure 7. Bipolar sensory cell with an apical cilium extending from a narrow short neck on a perikaryon with a short axon (a) having a long terminal bifurcation. Figure 29 has a camera lucida drawing of a similar cell. Scale bar = 3 μm.

Figure 8. Multipolar sensory cell with an apical cilium arising from a short neck, which forms one of three processes arising from a triangular perikaryon. Scale bar = 3 μm.

ium and a 7–9-μm-long neck (Fig. 9); (3) a 5-μm-long cilium, a 5–6-μm-long neck, and a pair of complexly branched basal processes (Fig. 10); and (4) a 3–5-μm-long cilium, a 6-μm-long neck, and several processes emanating from the base of the perikaryon (Fig. 11). The first two cell types have 3–5-μm-wide perikarya and a pair of simple basal processes, whereas the processes of the latter two types are more complex. The cells range in length from 11 to 124 μm (Table I).

Unipolar ganglion cells

Unipolar ganglion cells: single axon extending directly from the perikaryon, which lacks a cilium. The perikaryon variously appears as round (Fig. 12) or elongate (Fig. 13) with a slender axon or elliptical (Fig. 14) with a branched terminal and several short, twig-like processes. The perikarya were 9–10 μm wide. The cells ranged in length from 31 to 71 μm (Table I).

Bipolar ganglion cells

Bipolar ganglion cells: short or long processes extending from opposite sides of the perikaryon. Morphological types include (1) a short, 2–4-μm-long, perikaryal cilium with two lamellar processes (Fig. 15); (2) tiny cells with short, straight isopolar processes (Fig. 16); (3) asymmetrical cells with one short knobbed (Fig. 17) or tapered (Fig. 18) and one long process; (4) cells with one simple and one complexly branched process (Fig. 19); (5) cells with an elongated perikaryon that is 10–12 μm long and 5–7 μm wide with thick bifurcated processes (Fig. 20). The cells range in length from 39 to 210 μm (Table I).

Multipolar ganglion cells

Multipolar ganglion cells: three or more processes extending directly from the perikaryon. A few multipolar ganglion cells, all tripolar, have a perikaryal cilium (not shown by SEM). Morphological variations of ganglion cells include (1) tripolar ganglion cells with three similar processes (Fig. 21); (2) three dissimilar processes (Fig. 22); (3) round neurons with multiple short processes radiating from a central perikaryon (Fig. 23); (4) round perikarya with several dendrite-like processes and a 25-μm-long axon-like process with terminal branches (Fig. 24); (5) cells with irregular shapes and long slender (Fig. 25), basally webbed (Fig. 26), lamellar (Fig. 27), or fused looplike processes (Fig. 28). The latter four cell types with bizarre shapes differ from ciliated epithelial cells, having apical microvilli and basal muscle cells containing myonemes. Multipolar ganglion cells range in length from 31 to 310 μm (Table I).

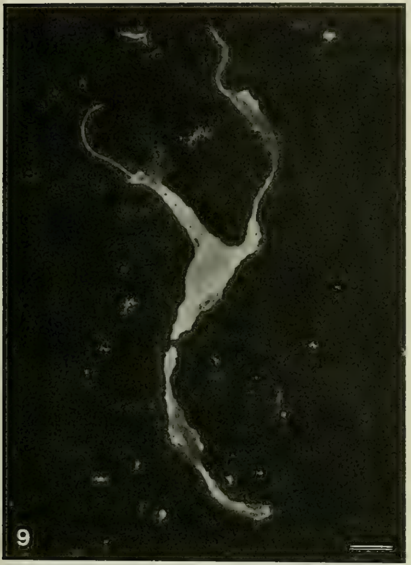
Quantitative analysis of neurons

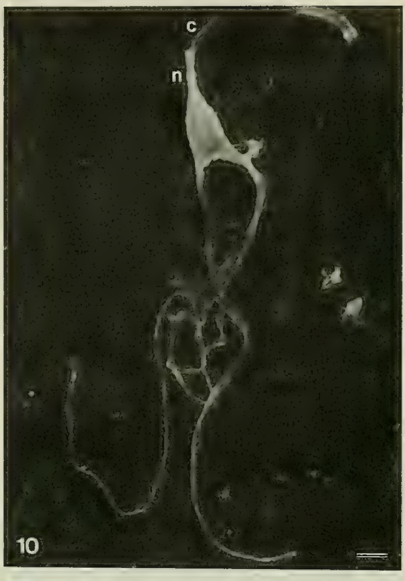
In four tentacles of the sea anemone *Calliactis parasitica,* 6217 neurons were counted, and 33 distinguishable forms were observed more than once (Fig. 29). Comparisons of fresh, fixed, and processed cells revealed similar types of neurons measuring 20–310 μm in length. In the rinses with sodium cacodylate buffer and 25% and 50% ethanol, a few bipolar and multipolar neurons with long processes (some more than 300 μm in length) were observed, measured, and photographed. In general, cells dissociated in collagenase were preserved better for SEM, whereas cells dissociated in elastase and ficin were better for cell counts because fewer were lost in the processing. Because the general shape and relative number of neurons dissociated from different tentacles were repeatable, we assumed that the morphological differences observed were consistent in the animal. Although some damage to processes and perikaryal shape changes may have occurred in the preparations, they did not alter our ability to categorize the major cell types. Therefore, we could distinguish sensory cells having an apical cilium from ganglion cells with or without a perikaryal cilium. Each group was categorized further as unipolar or bipolar based on the apical or middle location of its nucleus relative to its processes, whereas all neurons with three or more processes emanating from the perikaryon were classed as multipolar.

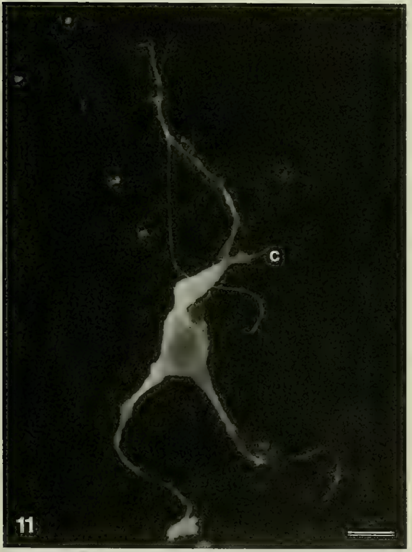
Statistical comparisons of different morphological categories of sensory and ganglion cells from four tentacles counted and averaged revealed significant differences (Fig. 30). Unipolar sensory cells were more frequent than unipolar ganglion cells ($P < 0.01$). In contrast, bipolar and multipolar ganglion cells were more frequent than bipolar and multipolar sensory cells ($P < 0.01$). Unipolar neurons constituted 27.05%, bipolar neurons 26.68%, and multipolar neurons 46.18% of the neurons counted in four tentacles. The number of cells counted for each type is given in Table I. The table also includes the percentage of total neurons counted for each type, and the percentage of each type within the sensory cell or ganglion cell categories.

Discussion

Neurons dissociated from *Hydra littoralis* and divided into those with an apical cilium (sensory cells) and those with no cilium or a perikaryal cilium (ganglion cells) were further categorized into unipolar, bipolar and multipolar types based on the number of identifiable processes extending from the perikaryon (Yu *et al.,* 1986). That classification scheme follows the vertebrate system for classifying morphological types of neurons according to the number of processes extending from the perikaryon (Williams and Warwick, 1975). What we have done in this study on neurons dissociated from tentacles of *Calliactis* and in a previous study of *Hydra* neurons (Yu *et al.,* 1986) is to extend the vertebrate morphological system of nerve cell classification to include sensory cells, in which the position of the perikaryon is apical, medial, or basal relative to the ciliated apex and axonal processes. A sensory







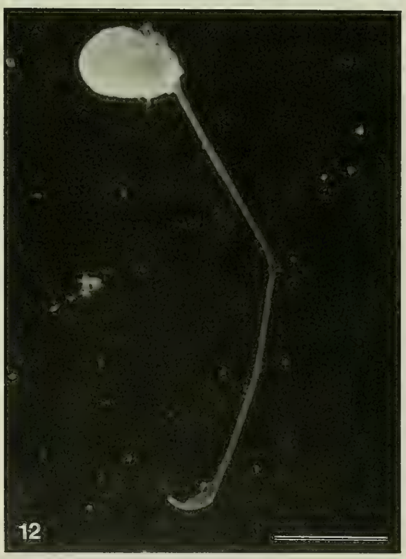
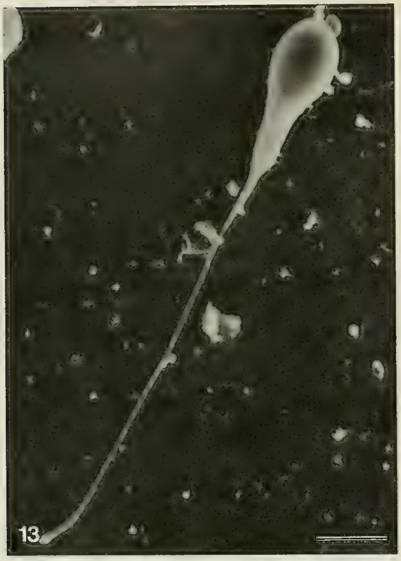


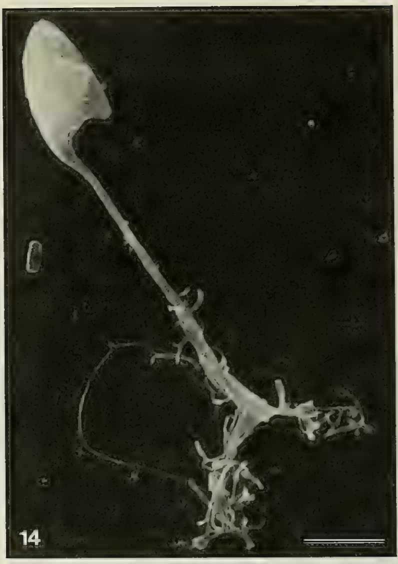
Figure 9. Tripolar (multipolar) sensory cell with a cilium arising from a long neck and two other oppositely directed, simple processes extending from a central pyriform-shaped perikaryon. Scale bar = 3 μm.

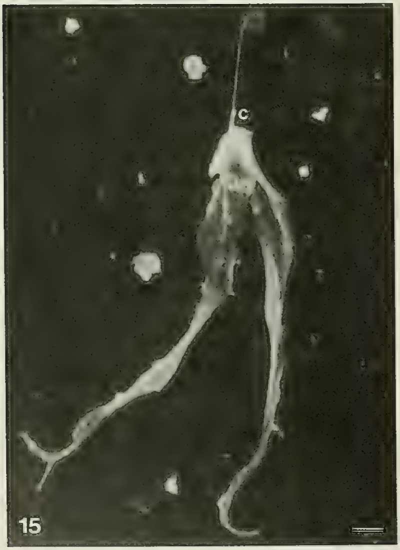
Figure 10. Multipolar sensory cell with a cilium (c) extending from a short neck (n) and two complexly branched processes extending from the central perikaryon. Scale bar = 3 μm.

Figure 11. Multipolar sensory cell with an apical cilium (c) extending from a long neck and several other processes extending from the perikaryon. Scale bar = 3 μm.

Figure 12. Unipolar ganglion cell with a round perikaryon and a simple slender axon. Scale bar = 3 μm.







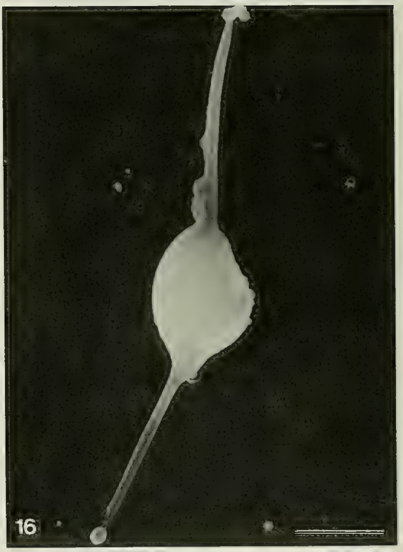
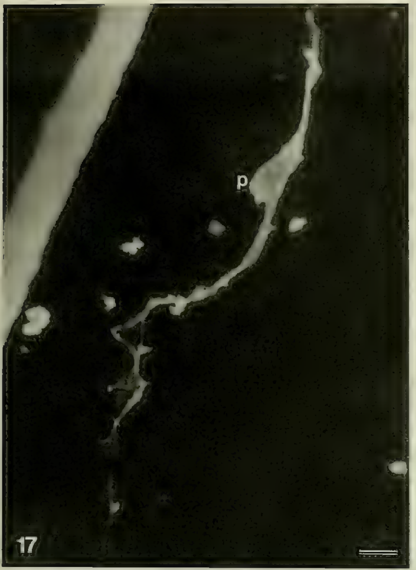
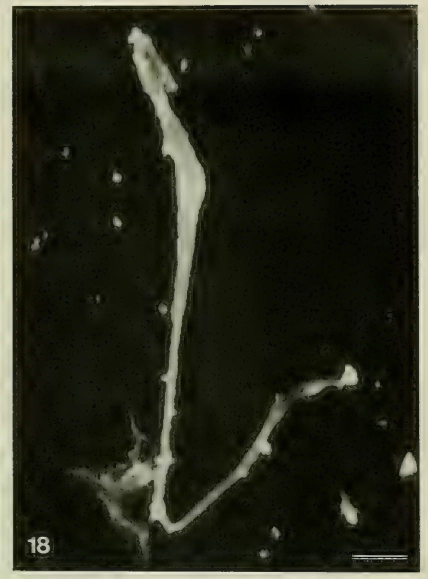
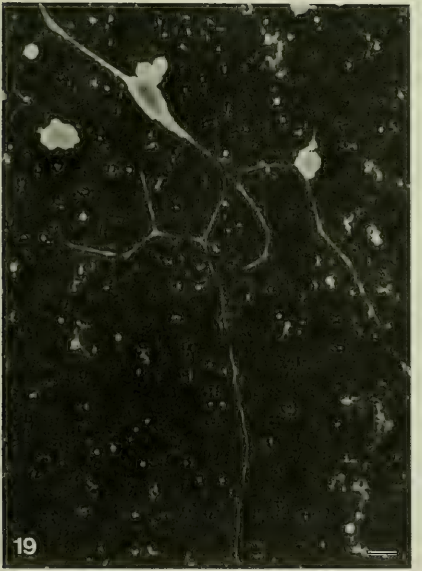


Figure 13. Unipolar ganglion cell with an elongate perikaryon and a simple slender axon. Scale bar = 3 μm.
Figure 14. Unipolar ganglion cell with an elliptical perikaryon and a short terminally branched axon. Scale bar = 3 μm.
Figure 15. Bipolar ganglion cell with a cilium (c) on the centrally located perikaryon from which two processes extend. Scale bar = 3 μm.
Figure 16. Small bipolar ganglion cell with short isopolar processes extending from the perikaryon. Scale bar = 3 μm.







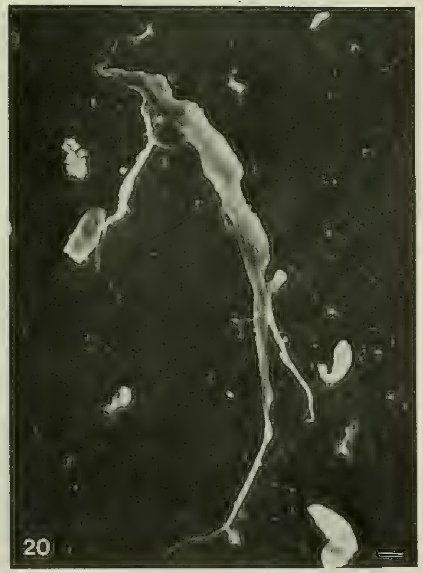
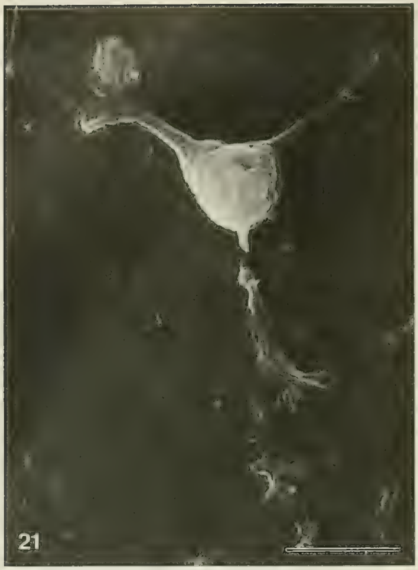


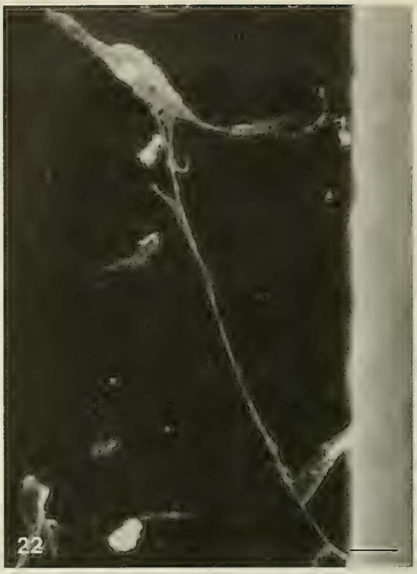
Figure 17. Asymmetrical bipolar ganglion cell with a terminal swelling or knob on the short process and an oppositely directed, long, simple process extending from the perikaryon (p). Scale bar = 3 μm.

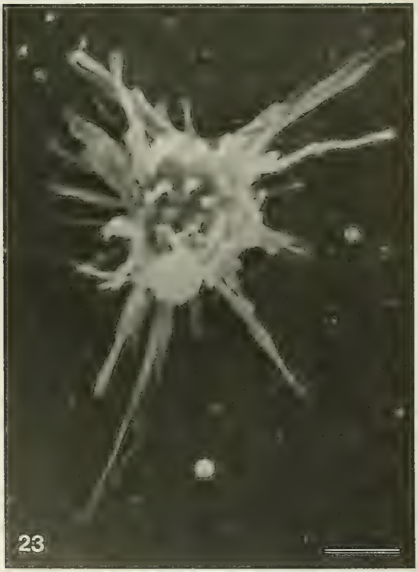
Figure 18. Asymmetrical bipolar ganglion cell with a short tapered process and a long simple process. Scale bar = 3 μm.

Figure 19. Asymmetrical bipolar ganglion cell with one simple process and an oppositely directed branched process extending from the perikaryon. Scale bar = 3 μm.

Figure 20. Bipolar ganglion cell with a central elongated perikaryon and oppositely directed lamellar bifurcated processes. Scale bar = 3 μm.







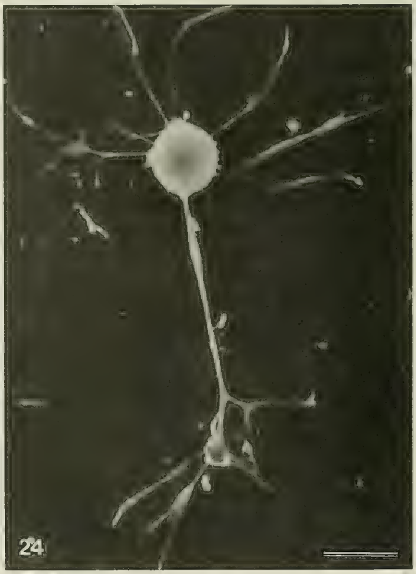
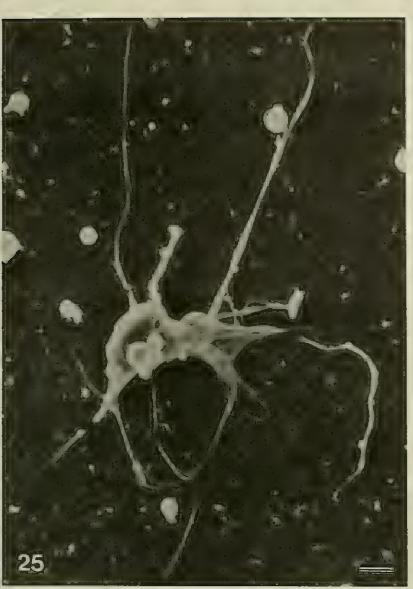


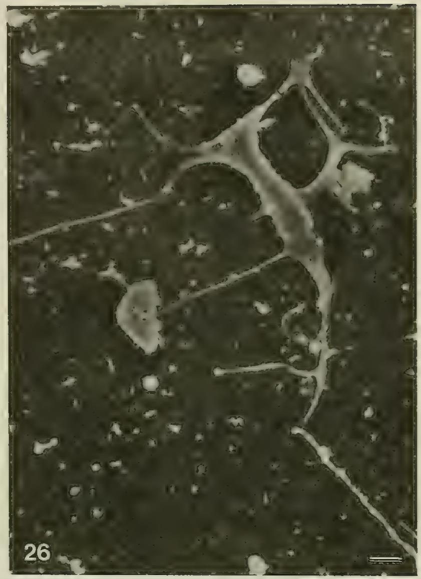
Figure 21. Tripolar (multipolar) ganglion cell with three equidistant processes extending from the centrally located perikaryon. Scale bar = 3 μm.

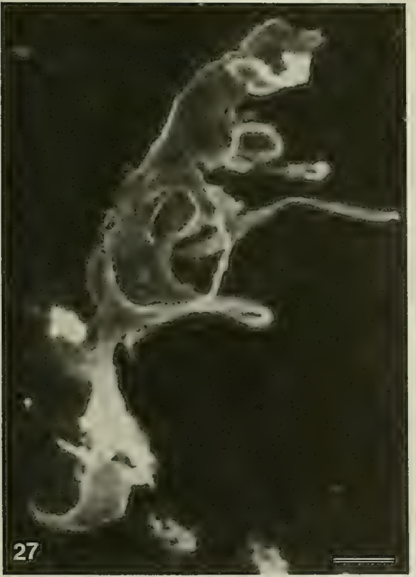
Figure 22. Multipolar ganglion cell with three asymmetrically arranged (one short and two long) processes extending from the perikaryon. Scale bar = 3 μm.

Figure 23. Multipolar ganglion cell with multiple short processes extending from a central round perikaryon. Scale bar = 3 μm.

Figure 24. Multipolar ganglion cell with several dendrite-like processes extending from the perikaryon and one long axon-like process with terminal branching. Scale bar = 3 μm.







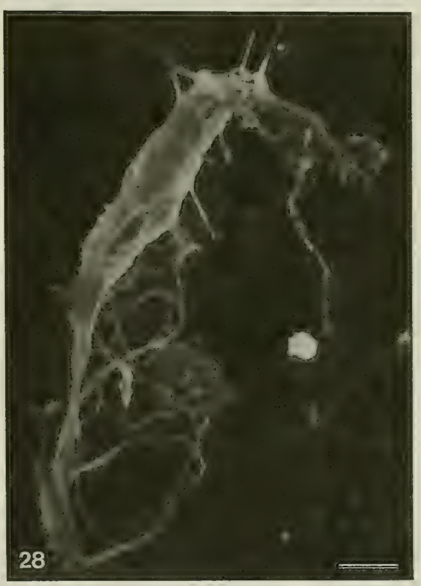


Figure 25. Multipolar ganglion cell with long slender processes extending from an irregularly shaped perikaryon. Scale bar = 3 μm.

Figure 26. Multipolar ganglion cell with an elongated perikaryon and some processes having thin web-like origins. Scale bar = 3 μm.

Figure 27. Multipolar ganglion cell with short processes extending from an elongate perikaryon. Scale bar = 3 μm.

Figure 28. Multipolar ganglion cell with an elongated perikaryon containing a cytoplasmic loop and numerous tangled processes. Scale bar = 3 μm.

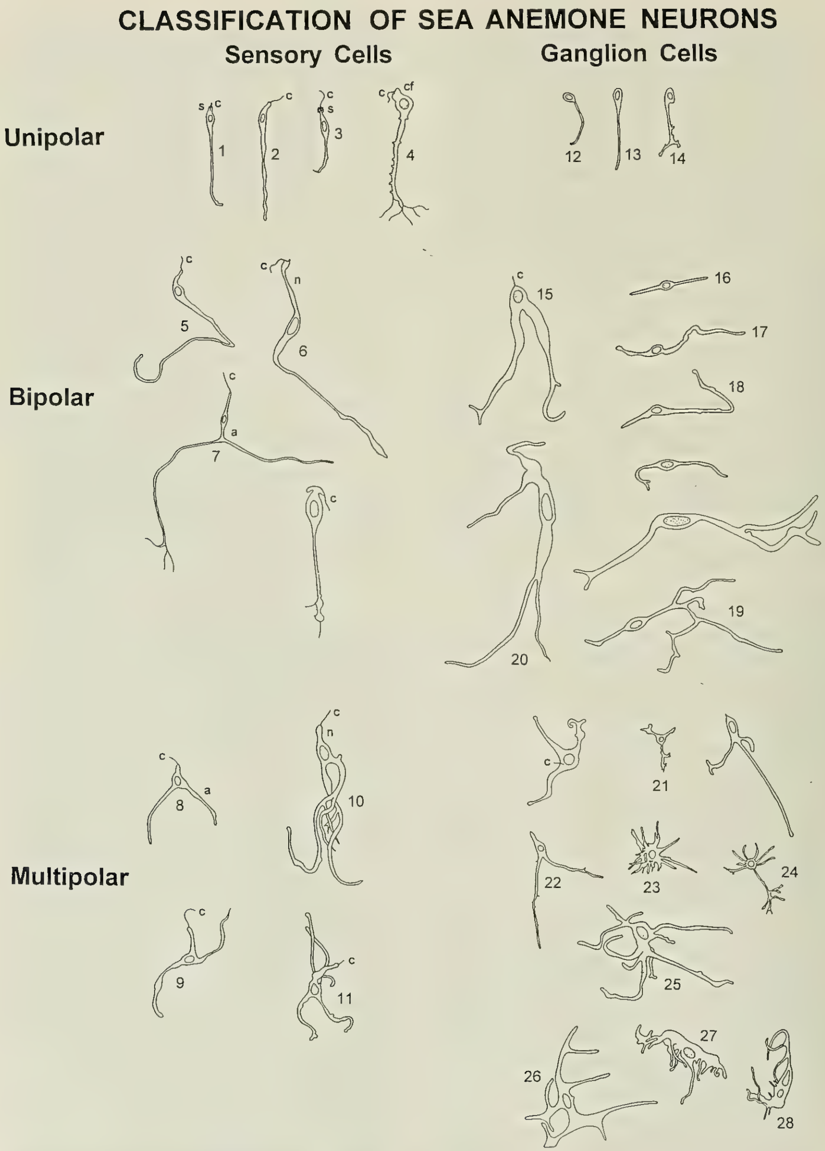

CLASSIFICATION OF SEA ANEMONE NEURONS
Sensory Cells
Ganglion Cells
Unipolar
Bipolar
Multipolar
s
c
1
c
2
c
s
3
c
cf
4
12
13
14
c
5
c
n
6
c
a
7
c
c
15
16
17
18
19
20
c
a
8
c
n
10
c
9
c
11
c
21
22
23
24
25
26
27
28

Figure 29. Classification scheme with drawings from either SEMS or light micrographs and some camera lucida tracings of representative neurons dissociated from tentacles of the sea anemone *Calliactis parasitica.* Unnumbered cells have no corresponding SEM figure. Drawings were reduced to make normal size comparisons except for #16, which could not be illustrated satisfactorily at a smaller size. Sensory cells have a visible apical cilium (c, left column); two types of ganglion cells have a cilium (c, right column) associated with the perikaryon. Some sensory cells also have stereocilia(s) either surrounding the cilium (#1) or ringing its base (#3), whereas some of the others have an apical cytoplasmic flare (cf), a short or long neck (n), and an axon (a) with or without terminal branching.

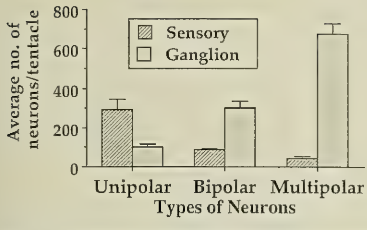


Figure 30. Histogram comparing average number of types of neurons per tentacle from a total of four tentacles counted.

cell with an apically located perikaryon resembles a unipolar ganglion cell, except that a cilium projecting into the environment extends from one pole of the perikaryon, whereas an axon projects from the opposite pole. Another category of sensory cells, based on the two types of ciliated bipolar sensory cells described in *H. attenuata* by Tardent and Weber (1976), has a short or long neck between the cilium and perikaryon. In the Tardent and Weber scheme, there are seven types (B_1–B_7) of bipolar neurons, only two of which are ciliated (B_3 and B_4). Because it is confusing to differentiate among these seven types, some of which may be sensory and others ganglion cells, we have chosen to simplify their scheme. Therefore, sea anemone neurons with an identifiable neck between an apical cilium and its perikaryon are simply termed bipolar sensory cells. The axons of these cells, which extend from a pole opposite to the neck region, may be short or very long, simple or branched. Sensory cells with more than one process extending from a perikaryon having a neck between it and the apical cilium are morphologically multipolar, *i.e.*, three or more arms extend from the perikaryon, one with a cilium. Multipolar sensory cells are easily distinguished from ciliated multipolar ganglion cells by the intervening neck on the sensory cells. Differences in position of the nuclei were shown in early drawings of isolated sensory cells in sea anemones (Hertwig and Hertwig, 1879; Grošlej; 1909), but no attempt was made to categorize sensory cells into different groups.

Both in this study and in previous studies of *Hydra* (Yu *et al.*, 1986; Westfall and Rogers, 1990), we have observed unipolar sensory cells with a bonnet-like apex, bipolar sensory cells with a curved neck and a posteriorly directed cilium, and pyriform-shaped multipolar sensory cells with basal nucleus and oppositely directed processes. Other investigators have reported apical ciliary-stereociliary complexes (ciliary cones) with projecting cilium in both sensory cells and nematocytes (see Fautin and Mariscal, 1991). In our cell dissociations, we have also found sensory cells with ciliary cones having a visible projecting cilium. Although the sensory cells portrayed in our classification scheme may not be representative of all sensory cells present in the animal, the morphological criteria for grouping sensory cells into unipolar, bipolar, and multipolar types can be applied to types not yet found.

One type of bipolar and multipolar neuron in *Calliactis* has a short perikaryal cilium like the one observed on similar types of ganglion cells isolated from *Hydra littoralis* (Westfall and Epp, 1985; Yu *et al.*, 1986). In *Hydra*, ganglion cells with a perikaryal cilium and neurosecretory-like granular vesicles were traced by TEM to synaptic contacts with other neurons and effector cells (Westfall, 1973a). Hence, they were considered to be morphologically multifunctional and perhaps representative of evolutionary precursors to specialized neurons in higher organisms. Until we locate and trace a ciliated ganglion cell to its synaptic contacts in the sea anemone, we cannot speculate on its functional morphology. However, synaptic loci have been observed in both epidermis and gastrodermis of sea anemone tentacles, and Antho-RFamide has been localized immunocytochemically in granular vesicles at two-way synapses in *Anthopleura* (Westfall and Grimmelikhuijzen, 1993). Both physiologic and immunofluorescent studies implicate neuropeptides as transmitters or modulators at neuromuscular junctions in *Calliactis parasitica* (Grimmelikhuijzen *et al.*, 1991). Therefore, the isolated nerve cells observed in this study are presumed to serve a purpose in sea anemones, such as having a role in feeding behavior.

Few studies have been done to quantify nerve cells in coelenterates or higher organisms. In *Hydra*, 40% of all nerve cells are concentrated in the region of the hypostome and tentacles (Bode *et al.*, 1973). Multipolar nerve cells account for 55%, bipolar cells for 42% and unipolar cells for 2% of the neurons in *Hydra* (Tardent and Weber, 1976). In the sea anemone *Calliactis parasitica*, we have found an average of 1554 ± 116 nerve cells per 6 mm length of tentacle, and of all the neurons observed in four tentacles, 46% were multipolar, 27% bipolar, and 27% unipolar. Multipolar ganglion cells were the most abundant type of neurons observed in sea anemone tentacles, whereas symmetrical bipolar neurons were dominant in tentacles of *Hydra* (Epp and Tardent, 1978).

Obviously, the procedure used to dissociate the cells plays an important role in the percentage of each nerve cell type obtained. In counting fixed and dehydrated cells, the number of a particular cell type may be influenced by the number of such cells that are retained on the coverslips and are not covered by the mesh of the locator grid. Despite these inadequacies, it is important to have a morphological basis for quantifying neurons in different species of the Cnidaria to understand more clearly how the first nervous systems evolved. According to Bullock and Horridge (1965), the simplest nervous systems consist entirely of bipolar and multipolar neurons. They speculate that unipolar neurons, which are dominant in the centralized nervous system of higher invertebrates, arise in the Platyhelminthes. In contrast, our study, along with a few other reports (Jha, 1965; Tardent and Weber, 1976; Epp and Tardent, 1978, Yu *et al.,* 1986; DellaCorte and McClure, 1991), has provided evidence for unipolar neurons in the Cnidaria, where nervous systems first appear. Moreover, we have demonstrated that not all bipolar and multipolar neurons are isopolar, *i.e.,* have similar processes, as was presumed previously for the simplest nervous systems. Some of the sea anemone ganglion cells can be categorized as multipolar heteropolar neurons with many processes at one pole, similar to those found in the brains of cephalopods, arthropods, and vertebrates (Bullock and Horridge, 1965).

In summary, our light and electron microscopic characterization of neurons dissociated from sea anemone tentacles has provided morphological criteria to classify these primitive nerve cells and to quantify the predominant types among them. These data may aid in the classification of other coelenterate neurons regardless of their size or function.

Acknowledgments

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Efferent Mechanisms of Discharging Cnidae: I. Measurements of Intrinsic Adherence of Cnidae Discharged From Tentacles of the Sea Anemone, *Aiptasia pallida*

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Abstract. Two kinds of cnida predominate in the tentacles of the acontiate sea anemones: spirocysts and microbasic mastigophore nematocysts. These cnidae discharge in response to appropriate mechanical and chemical stimulation. In this paper, we calculate the strengths of attachment between the tentacle and the capsules (= "tentacle adherence") of discharged spirocysts and mastigophores by measuring adhesive force and by determining the numbers of spirocysts and mastigophores discharged onto targets under conditions where the attachment of everted cnida tubules to the target (= "cnida adhesion") exceeds tentacle adherence. Under these conditions, the average contribution of individual cnidae to adhesive force is called the intrinsic adherence. The intrinsic adherence is a measure of the average frictional force required to dislodge the capsule of individual discharged cnidae from the tentacle. The intrinsic adherence of discharged mastigophores varies inversely, from 0.45 to 0.15 mgf (4.41 to 1.47 μN), with the number of discharged mastigophores. The larger values characterize mastigophores discharged by mechanically triggering nonchemosensitized tentacles, whereas the lower values characterize the intrinsic adherence of mastigophores discharged from chemosensitized tentacles. In contrast, the intrinsic adherence of discharged spirocysts is very low to insignificant. Thus, by comparison to mastigophores, spirocysts contribute little, if any, to adhesive force, and, by inference, do not directly secure captured prey to the tentacle. Our measurements indicate that penetrable prey are primarily secured to the tentacle by discharged mastigophores and by the inherent stickiness of the tentacle surface.

Introduction

Capture of prey

Active predators use remote sensing systems, such as vision or hearing, to detect and select prey over distance and to direct the neuromuscular movements needed to pursue and capture prey. Passive predators, on the other hand, do not usually select prey at a distance and do not pursue prey. They commonly rely upon local sensing systems, such as touch and chemical sensing, to detect and select prey in proximity and to elicit extremely rapid capture behaviors. Passive predation emphasizes rapid selection and responsiveness to the arrival of suitable prey. Cnidarians are obligate predators, and all, with the possible exception of the cubomedusae, are passive predators.

Feeding in sessile cnidarians, such as sea anemones, consists of sequentially linked prey capture, feeding response, and ingestion behaviors. In general, prey capture is initiated by appropriate stimulation of chemo- and mechano-receptors, and is culminated by cnida-mediated retention of prey to the tentacle (Thorington and Hessinger, 1988b). The subsequent feeding response, also activated by chemical and mechanical stimuli originating from the prey, is a coordinated series of neuromuscular movements that transfers captured prey to the oral disk and opens the mouth for ingestion to occur (Lenhoff and Heagy, 1977).

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Abbreviations: CSCC, cnidocyte/supporting cell complex; S_t, tentacle stickiness; i_s, intrinsic adherence of spirocysts; i_m, intrinsic adherence of mastigophore nematocysts.

Afferent mechanisms regulate cnida discharge

While anemones and other cnidarians employ cnidae in various ways, cnidae are primarily used to capture prey (Ewer, 1947). We define mechanisms that regulate the initiation of cnida discharge (*i.e.,* receptor-effector coupling) as cnida afferent mechanisms. These mechanisms control the discharge of cnidae through the interplay of both local receptors associated with the cnidocytes that trigger discharge (Watson and Hessinger, 1988, 1991) and remote receptors that modulate cnidocyte responsiveness (as reviewed in Thorington and Hessinger, 1988b). In acontiate sea anemones, such as *Aiptasia pallida* and *Haliplanella luciae,* the functional and structural unit of cnida discharge is the cnidocyte/supporting cell complex (CSCC). In these anemones, two or more supporting cells surrounding each cnidocyte appear to bear at least one kind of chemoreceptor (Watson and Hessinger, 1988) and mechanoreceptor (Watson and Hessinger, 1991). When appropriately stimulated, two or more supporting cells surrounding each cnidocyte act upon the cnidocyte to initiate discharge.

We have identified three types of CSCC: Types C, B, and A (Thorington and Hessinger, 1990). Type C CSCCs are induced to discharge by sufficient physical contact. Type B CSCCs are induced to discharge by a combination of both contact and chemical stimulation. Chemoreceptors for free and conjugated N-acetylated sugars (*e.g.,* N-acetylneuraminic acid, NANA) and for certain amino compounds predispose contact-sensitive mechanoreceptors to trigger discharge (Thorington and Hessinger, 1988a, 1990). In addition, vibration-sensitive mechanoreceptors are associated with Type A CSCCs (Watson and Hessinger, 1989) and all three kinds of receptor are involved. Type A CSCCs discharge their cnidae in response to triggering contact stimuli following sensitization by vibrations at specific frequencies to which they can bidirectionally tune by stimulating antagonistic NANA (Watson and Hessinger, 1991) and proline chemoreceptors (Watson and Hessinger, 1994a).

Efferent mechanisms regulate cnida function

After the initiation of discharge, efferent mechanisms regulate the use of cnidae. As used in this report, the terms cnida adhesion, tentacle adherence, intrinsic adherence, and adhesive force have specific meanings associated with aspects of efferent mechanisms. Cnida adhesion refers to the interaction between everted cnida tubules and targets, and is measured as the force needed to separate the cnidae from the target. Tentacle adherence refers to the interaction between capsules of discharged cnidae and the surrounding tentacle to which they are anchored, and is measured as the force needed to remove discharged cnidae from the tentacle. The tentacle adherence of an individual cnida is called the intrinsic adherence. Intrinsic adherence is a measure of the frictional force required to dislodge the capsule of the discharged cnida from a tentacle. Adhesive force is a measure of the applied force needed to separate a target from the tentacle and is the sum of contributions arising from the discharged cnidae and the inherent stickiness of the tentacle mucus (Thorington and Hessinger, 1988a, 1990). When cnida adhesion exceeds tentacle adherence, contributions of discharged cnidae to adhesive force provide a measure of tentacle adherence. These relationships are shown diagramatically in Figure 1 and described later in Equation 1. Additional efferent mechanisms include the penetration of the target by everting nematocyst tubules and the delivery of nematocyst venom to the target. It is not yet known whether cnida efferent mechanisms are biologically regulated as are cnida afferent mechanisms. It is likely, however, that tentacle adherence is regulated, since prey attached to the tentacle by cnidae during capture are subsequently released from the tentacle during ingestion.

On the basis of the adhesive property of everted spirocyst tubules and the penetrating and venomous properties of mastigophores (Mariscal, 1974), it has been commonly inferred that penetrant nematocysts principally envenomate and immobilize prey, while spirocysts principally attach stung prey and inert substrates to the tentacles (McFarlane and Shelton, 1975; also, "Spirocysts are adhesive organelles which function both in prey capture and substrate attachment," Mariscal *et al.,* 1977). In other words, it has been commonly inferred that the intrinsic adherence of spirocysts exceeds that of penetrant nematocysts. On the other hand, we have recently presented qualitative evidence that mastigophore nematocysts predominate over spirocysts in securing penetrable targets to the tentacles (Thorington and Hessinger, 1990). However, until now, quantitative evidence for inferring the roles of mastigophores and spirocysts in the retention of prey has been limited to counting the numbers of discharged cnidae, but determination of their intrinsic adherence has been lacking.

As a part of our on-going investigation into the mechanisms that regulate cnida discharge, we have developed methods to quantitate discharging cnidae under conditions in which the eliciting stimuli and the target surfaces are defined. We now measure the tentacle adherence of cnidae under conditions in which cnida adhesion does not influence adhesive force (*i.e.,* when cnida adhesion exceeds tentacle adherence). From the measurements of tentacle adherence using monoclonal, laboratory-reared sea anemones (*Aiptasia pallida*), we infer the relative roles of mastigophores and spirocysts in securing captured prey. Our findings indicate that as long as mastigophores are

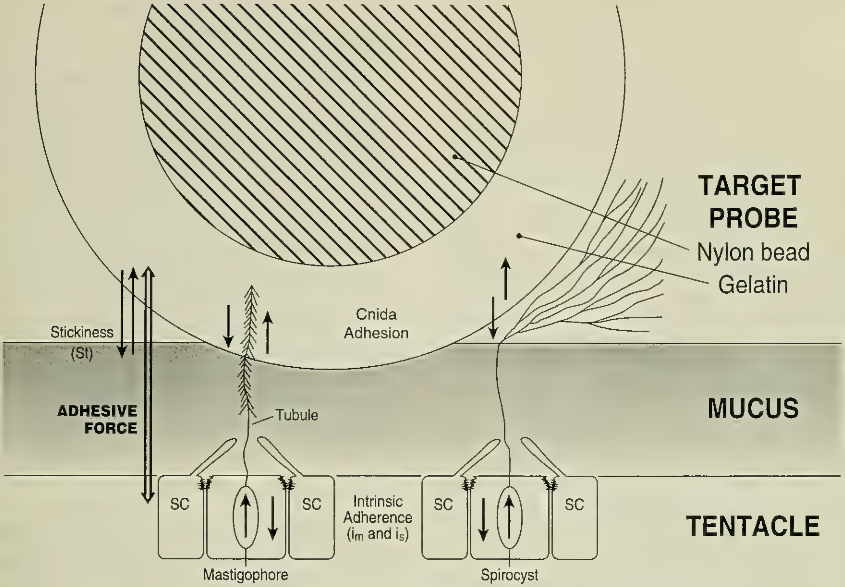

Figure 1. Relationship of adhesive force to cnida adhesion, intrinsic adherence, and tentacle stickiness. Double arrow represents the net opposing forces operating between the target probe and the tentacle, which is termed adhesive force. The individual opposing forces constituting adhesive force are: (i) cnida adhesion, operating between the target and the everted cnida tubule; (ii) intrinsic adherence (*i.e.*, i_m and i_s for mastigophore and spirocyst intrinsic adherence, respectively), operating between the tentacle and the cnida capsule; and (iii) stickiness (S_t), operating between the target and the surface mucus of the tentacle. When cnida adhesion exceeds tentacle adherence (the sum of all contributing intrinsic adherences), then the adhesive force is a function of the tentacle stickiness plus the sum of the intrinsic adherences of all the involved mastigophores and spirocysts. Diagram not drawn to scale. SC, supporting cell.

able to penetrate the surface of prey, cnida-mediated attachment of prey to tentacles is provided predominantly by mastigophore nematocysts, not by spirocysts. Thus, the function of spirocysts is in need of reevaluation.

MATERIALS AND METHODS

Maintenance of sea anemones

Monoclonal sea anemones (*A. pallida,* Carolina strain) were maintained individually in glass finger bowls containing natural seawater at 24 ± 1°C. Filtered seawater was obtained from the Kerckoff Marine Laboratory of the California Institute of Technology at Corona del Mar, California. Anemones were fed daily with freshly hatched brine shrimp nauplii (*Artemia salina*) and washed 4–6 h after feeding. Animals were kept on a 12/12 h photoperiod using white fluorescent lights at an intensity of 5.5 klux ($66\ \mu\ \mathrm{Es}^{-1}\ \mathrm{m}^{-2}$).

Experimental animals and test solutions

Animals of the same size were starved 72 h prior to experimentation and kept under continuous fluorescent light at 4.5 klux ($54\ \mu\ \mathrm{Es}^{-1}\ \mathrm{m}^{-2}$) during the last 48 h of starvation. Exposure to continuous light enhanced uniformity of anemone behavior and cnidocyte responsiveness. Just prior to use, the animals were gently rinsed with fresh seawater to remove soluble wastes and the medium was exchanged with the solution to be tested. The animals were permitted to adapt to the change of medium for 10 min before cnidocyte responsiveness was measured. Test solutions of various known chemosensitizers, N-acetylneuraminic acid (NANA), glycine, histamine, and proline (Sigma, St. Louis, MO) were prepared in natural, filtered (Type 1, Whatman) seawater adjusted to pH 7.62 with 1 *N* HCl or NaOH. Calcium-free artificial seawater (Ca-free ASW) consisted of 439 m*M* NaCl, 8.9 m*M* KCl, 23 m*M* $MgCl_2$, 22.5 m*M* $MgSO_4$, 4.6 m*M* $NaHCO_3$, and

1 m*M* EGTA (ethyleneglycoltetraacetic acid) at final pH of 7.62.

Measurements of cnidocyte responsiveness

Three measures of cnidocyte responsiveness were used: (i) measurement of adhesive force; (ii) enumeration of discharged mastigophores by microscopic examination; and (iii) enumeration of discharged spirocysts by enzyme-linked lectin sorbent assay (ELLSA).

Adhesive force. Adhesive force is a collective measure of both cnida-mediated and cnida-independent forces that act to retain a target surface to the tentacle. The measurement of adhesive force has been described in detail elsewhere (Thorington and Hessinger, 1988a). It employs a gravitometrically calibrated strain gauge to which is attached a gelatin-coated nylon bead of defined size (the test probe or target), which is touched to the distal third of a primary tentacle on an anemone submerged in seawater. The chemosensitizing agent may be presented to the anemone either in the seawater or in the gelatin-coating of the probe. Physically contacting the tentacle with the test probe mechanically triggers local discharge of cnidae. The everting tubules of the nematocysts and of the spirocysts, respectively, penetrate and stick to the gelatin coating. As the probe is pulled away, the discharged cnidae resist separation from the tentacle, causing an equal and opposite force to be exerted on the strain gauge. The measured adhesive force, which is recorded on a strip chart recorder, is that required to separate the probe from the tentacle. Adhesive force measurements are expressed in hybrid units of milligram-force (mgf), rather than newtons (or dynes), since there is negligible acceleration, thereby making contributions from Newton's Second Law insignificant (Miller, 1959).

Enumeration. After being used to measure adhesive force, the gelatin coating of the probe tips are enzymatically digested to release the discharged mastigophore nematocysts. The highly refractile mastigophores, which are resistant to proteolysis, are then counted with a microscope from the bottoms of flat-bottomed microtiter wells. The procedures used to remove and count the nematocysts have been published (Geibel *et al.,* 1988).

Enzyme-linked lectin sorbant assay (ELLSA). To determine the number of spirocysts discharged onto test probes, we used an enzyme-linked lectin sorbent assay, the details of which have been published by Thorington and Hessinger (1990). This assay is based upon the high-affinity binding of conjugated N-acetylated sugars to the everted tubules of discharged spirocysts on the surface of test probes. The assay involves colorimetric measurement of bound peroxidase activity using a microtiter plate spectrophotometer following the sequential treatment of test probes with solutions of asialomucin and *Vicia villosa* lectin/peroxidase conjugate.

Collection and analysis of cnidocyte response data

Separate animals were exposed to different concentrations of chemosensitizers in seawater. Six probes, one per tentacle, were used on each animal to measure adhesive force and to enumerate discharged mastigophores and spirocysts. Daily experimental means were calculated from these measurements and replicate experiments were performed on four days. Data points represent the mean of the daily experimental means ($n = 4$). In some cases, where indicated, previously published experimental values (Thorington and Hessinger, 1990) are used to calculate the contributions of individual cnidae to adhesive force measurements. Linear regression analysis and determination of extrapolated values for intrinsic adherence were performed with a computer-assisted graphics and data-formatting program (Dorgan and Hessinger, 1984).

Contributions to adhesive force

Adhesive force is a composite measure of contributions from discharged mastigophores, discharged spirocysts, and the stickiness of the tentacles due to its mucus layer. Under conditions where cnida adhesion exceeds tentacle adherence, the contribution of each kind of cnida to adhesive force is the product of the number of cnidae discharged and the force required to remove one such cnida from the tentacle (*i.e.,* intrinsic adherence). These relationships may be summarized by the following equation:

$$\text{Adhesive force} = S_t + (i_m)(n_m) + (i_s)(n_s) \qquad \text{(Equation 1)}$$

where:

S_t is the stickiness of a tentacle to a test probe in the absence of discharging cnidae, expressed in mgf;
i_m is the intrinsic adherence of mastigophores, expressed as mgf/mastigophore;
n_m is the number of discharged mastigophores retained on the probe;
i_s is the intrinsic adherence of spirocysts, expressed as mgf/spirocyst;
n_s is the number of discharged spirocysts retained on the probe.

Stickiness of tentacles (S_t). We sought to determine the contribution of tentacle surface mucus to adhesive force by measuring adhesive force in the absence of discharging cnidae. Because external calcium is required for cnida discharge in sea anemones (Blanquet, 1970; Lubbock and Amos, 1981; Thorington and Hessinger, 1992; Watson and Hessinger, 1994b), we attempted to inhibit discharge by either pre-treating anemones for 90 min in Ca-free

ASW or by following the pre-incubation in Ca-free ASW or ASW with 10% formalin for 5 min at 4°C or at room temperature before measuring adhesive force and cnida discharge. Adhesive force measurements were made using probes coated with either 30 or 75% (w/v) gelatin. After measuring adhesive force, the number of discharged mastigophores and spirocysts adhering to the test probes was determined. The data are averages of several experiments with six replicate measurements for each experiment.

Intrinsic adherence of spirocysts (i_s). To calculate the value of i_s, we measured adhesive force in the absence of contributing mastigophores using 75% gel-coated probes. Since the relatively hard surface of 75% probes makes them impenetrable to the everting tubules of most discharging mastigophores (Thorington and Hessinger, 1990), the few attached mastigophores are assumed to make no contribution to the measured adhesive force. The value of i_s was calculated from the following, modified version of Equation 1, using 75% probes in the presence of various chemosensitizers:

$$\text{Adhesive force} = S_t + (i_s)(n_s) \qquad \text{(Equation 2)}$$

Experiments were performed at the concentrations of sensitizers giving maximal effects (EC_{100}) and half-maximal effects ($K_{0.5}$) on dose-responses. Sensitizers exhibiting bi- or tri-modal dose-responses (Thorington and Hessinger, 1990) were tested at each of the EC_{100} doses and the resulting values of i_s were averaged.

Intrinsic adherence of mastigophores (i_m). Values of i_m were calculated from equation 1 using the measured adhesive force, the numbers of discharged spirocysts (n_s) and mastigophores (n_m) for each tested dose of sensitizer, as well as the values of S_t and i_s, which were separately determined.

Determination of mastigophore size

Mastigophore nematocysts were discharged with 30% gel-coated probes following exposure of the Carolina strain anemones to EC_{100} doses of different sensitizers. The lengths and widths of the capsules were measured using phase-contrast optics at a final magnification of 320× (Nikon Diaphot 20 DL objective) and an ocular reticule calibrated from a stage micrometer. In addition, size comparisons of undischarged mastigophore nematocysts were made on microscope slides from squash preparations of tentacles from Miami and Carolina strains of anemones. Length and width measurements of 100 capsules were averaged for each strain.

Statistical analysis

One-way analysis of variance (ANOVA) with the Scheffe t-test was used to calculate *P*-values. A P-value $\leq$ 0.05 was considered to be statistically significant unless otherwise noted. Data are expressed as mean ± standard error.

Results

Tentacle stickiness (S_t)

To determine the contributions of discharging cnidae to measurements of adhesive force, it is necessary to know first the cnida-independent contributions to adhesive force, which we term tentacle stickiness (S_t). Tentacle stickiness can be measured directly from test probes in the absence of discharging cnidae. We attempted to block the discharge of cnidae and measure tentacle stickiness in several ways (Table I).

Effects of cold on cnida discharge. By chilling the ASW from room temperature to 4°C, the discharge of spirocysts onto probes coated with 30% gelatin was reduced by two thirds, the discharge of mastigophores by four fifths, and the adhesive force by about 23% (Table I). With 75% probes, chilling reduced the discharge of spirocysts onto probes by two thirds, while the number of discharged mastigophores retained on the probe was unchanged, but low, since the probes were too hard for discharging mastigophores to penetrate (Thorington and Hessinger, 1990). The adhesive force measured by 75% probes also remained unchanged, which is qualitatively consistent with our view that mastigophores, and not spirocysts, are the predominant cnida contributing to adhesive force (Thorington and Hessinger, 1990). At 4°C, 75% probes recorded significantly lower adhesive force than 30% probes despite equal numbers of discharged mastigophores and 67% more spirocysts. This suggests that the mastigophores on 30% probes contribute to adhesive force, whereas mastigophores on 75% probes do not.

Effects of low calcium on cnida discharge. Anemones, exposed for 90 min to Ca-free ASW at 4°C or exposed to 10% formalin in Ca-free ASW for 5 min at 4°C following 90 min preincubation in cold Ca-free ASW, exhibited no significant discharge of cnidae, but significant and equivalent adhesive forces (Table I). Formalin fixation does not appear to alter tentacle stickiness neither by changing the stickiness of tentacle mucus nor by altering the interaction of the tentacles with gelatin-coated test probes due to unreacted aldehyde groups, as shown by comparing the adhesive force for Ca-free ASW at 4°C with and without 10% formalin.

Effects of formalin on cnida discharge. To determine the intrinsic adherence of cnidae using Equations 1 and 2, it is first necessary to obtain a value of S_t at room temperature in ASW; the conditions used during subsequent experiments. Blocking cnida discharge with cold or with Ca-free ASW does not meet these requirements. We,

Table I

Cnida-independent contribution to adhesive force

	30% Probes			75% Probes		
Treatment	Mastig.[b]	Spiro.[c]	Ad. force[d]	Mastig.[b]	Spiro.[c]	Ad. force[d]
ASW:[a]						
22–24°C	88 ± 12	93 ± 3	56 ± 1	19 ± 3	170 ± 32	34 ± 1
4°C	19 ± 2	36 ± 6	43 ± 5	19 ± 0.2	60 ± 14	34 ± 2
Ca-free ASW:[e]						
4°C	4 ± 2	0	34.0 ± 0.7	5 ± 3	0	28.0 ± 3
4°C, formalin	1 ± 0.5	0	34.9 ± 0.5	2 ± 0.8	0	28.2 ± 7
ASW:[f]						
22–24°C, formalin	0	0.7 ± 0.1	34.6 ± 0.7	0.4 ± 0.1	0.5 ± 0.1	32.9 ± 0.3

[a] Anemones were pretreated in ASW at either room temperature (22–24°C) or 4°C for 10 min before being tested with either 30% or 75% probes (n = 20).

[b] Mean number of discharged mastigophores ± SEM.

[c] Mean number of discharged spirocysts ± SEM.

[d] Mean adhesive force, mgf ± SEM.

[e] Anemones were pretreated in Ca-free ASW for 90 min before being tested or before being exposed to 10% formalin in Ca-free ASW for 5 min at 4°C and then tested (n = 31).

[f] Anemones were pretreated in ASW for 90 min and then exposed to 10% formalin in ASW for 5 min at room temperature (22–24°C) before being tested (n = 22).

therefore, used formalin-fixation in ASW at room temperature both to preserve tentacle stickiness while also blocking cnida discharge (Table I). Thus, the measured stickiness of tentacles (S_t) to 30% probes in ASW at room temperature averages 34.6 mgf (339.1 μN), which is only 5% higher than to 75% probes (Table I).

Intrinsic adherence of spirocysts (i_s)

The contribution of discharging spirocysts to adhesive force was measured directly using 75% gelatin-coated probes. Such probes permit the adhesive tubules of discharging spirocysts to attach to the probe surface, but prevent most of the everting tubules of discharging mastigophores from penetrating (Table II; Thorington and Hessinger, 1990). Using EC_{100} doses of glycine, histamine, proline, and NANA (Thorington and Hessinger, 1990), we measured the adhesive force and counted the numbers of mastigophores and spirocysts attached to 75% probes (Table II).

The number of mastigophores retained on 75% probes averaged approximately 20% of the number retained on 30% probes under similar test conditions (Thorington and Hessinger, 1990). If we assume that the contribution to adhesive force of the relatively few, retained mastigophores on 75% probes is negligible (Table I), then the maximum contribution to adhesive force of the discharged spirocysts can be calculated from Equation 2 by subtracting the contribution of S_t (*i.e.*, 32.9 ± 0.3 mgf or 322.4 μN; Table I). Dividing the spirocyst contribution to adhesive force by the number of discharged spirocysts retained on the probe yields the intrinsic adherence of spirocysts (i_s) for each test condition (Table II). The value of i_s for spirocysts discharged from non-chemosensitized animals (*i.e.*, from Type C CSCCs) is 0.006 mgf (0.059 μN), which is lower than the average value of 0.016 mgf (0.157 μN) from chemosensitized animals (*i.e.*, from both Type B and Type C CSCCs in a ratio of about 1:1; Table II). Furthermore, the value of i_s appears to be independent of the kind of chemosensitizing agent used.

Intrinsic adherence of mastigophores (i_m)

Because we have no way of nulling the contribution of discharging spirocysts or of specifically blocking only spirocyst discharge, the contribution of discharging mastigophores cannot be directly measured as it was for spirocysts. Thus, it is necessary to calculate the contribution of discharging mastigophores using indirect approaches. This can be done by (i) relating the number of discharged mastigophores to adhesive force and graphically determining the mean intrinsic adherence from the slope of such a relationship, and by (ii) calculating i_m from equation 1 after obtaining values for all the other parameters of the equation.

Mean intrinsic adherence. The mean intrinsic adherence of discharging mastigophores can be approximated from the slope of the measured adhesive force plotted against the number of discharged mastigophores for sensitizing doses of chemosensitizers (Fig. 2). The number of discharged mastigophores correlates directly and linearly to adhesive force. The least-squared, best-fit line for these data, when extrapolated, intercepts the abscissa at 39.9 mgf (391.0 μN). We interpret this intersection to indicate that the measured adhesive force consists of two components: one, up to about 39.9 mgf, independent of discharging mastigophores, including contributions from the stickiness of tentacles (34.6 mgf or 339.1 μN; Table I) and from discharging spirocysts; and the other dependent upon the discharging mastigophores. From the slope of this extrapolated line, we calculate that the mean intrinsic adherence of all discharging mastigophores (*i.e.*, from both Type B and Type C CSCCs) is 0.18 mgf (1.76 μN); a value more than ten times that of the average value of i_s (Table II).

Calculating i_m from Equation 1. Using the values determined for the inherent stickiness of the tentacle (S_t; Table I) and the average intrinsic adherence of the spirocysts (i_s; Table II) along with previously published values of adhesive force and of the total number of discharged spirocysts (n_s) and mastigophores (n_m) from dose-responses to various chemosensitizers (Thorington and Hessinger, 1990), it is possible to calculate the values of i_m (Eq. 1). We find that i_m varies three-fold and inversely with changes in the number of discharging mastigophores, regardless of chemosensitizer used. Overall, the values for i_m vary nonlinearly from a maximum of about 0.45 mgf (or 4.41 μN)/cnida to a minimum of about 0.15 mgf (or 1.47 μN)/cnida over the range of 50 to 250 discharged mastigophores, respectively (Fig. 3A). For each of the chemosensitizers tested, with the possible exception of proline (Fig. 3E), the shape of the i_m-discharge relationship is a declining hyperbola (Fig. 3B-D). The highest i_m values correspond to mastigophores discharged from Type C CSCCs in control anemones (Fig. 3, solid symbols), while lower i_m values correspond to mastigophores discharged from a combination of Type B and Type C CSCCs in chemosensitized anemones. The declining hyperbolic shape of the i_m-discharge relationship suggests either physical changes to the tentacle or mastigophores during discharge, which affect i_m values, or a heterogeneity in i_m values among different kinds of responding CSCCs.

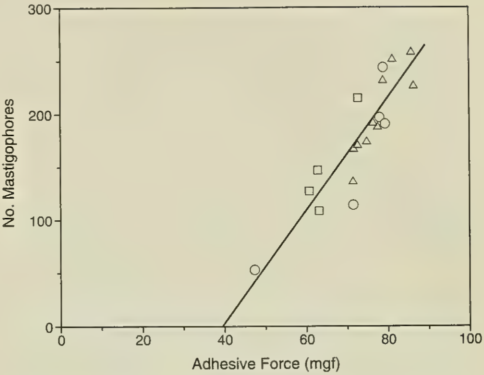

Figure 2. Number of discharged mastigophores correlated to measured adhesive force. The number of mastigophores discharged and the measured adhesive force (mgf) are indicated on the ordinate and abscissa, respectively, from anemones exposed to sensitizing doses of NANA (triangles), glycine (circles), and histamine (squares). The extrapolated least-squares fit line intercepts the abscissa. Each data point is the mean of four experiments, each comprised of six tentacle readings (R = 0.89).

Table II

Intrinsic adherence of spirocysts using 75% probes

Agonist	Mastig.[a] (n_m)	Ad. force[b] (mgf)	Spiro.[c] (n_s)	Intr. adh.[d] (i_s)
Control[e]	19 ± 3	34 ± 1	170 ± 32	0.006
Glycine (5.4 × 10^{-12} M)[f]	1 ± 0.4	39 ± 3	371 ± 49	0.016
Histamine[f] (10^{-12} M)	14 ± 8	32 ± 2	308 ± 13	−0.003
(9.7 × 10^{-10} M)	28 ± 4	37 ± 7	214 ± 69	0.019
Proline[f] (3.2 × 10^{-9} M)	50 ± 6	42 ± 3	343 ± 22	0.026
(1.7 × 10^{-7} M)	29 ± 10	39 ± 4	382 ± 35	0.016
NANA[f] (5 × 10^{-9} M)	19 ± 3	38 ± 3	281 ± 27	0.018
(8 × 10^{-8} M)	18 ± 5	39 ± 2	266 ± 56	0.023
(10^{-5} M)[g]	24 ± 2	41 ± 4	368 ± 34	0.022
			Average	0.016 ± 0.002

[a] Mean number of discharged mastigophores ± SEM (n = 5).

[b] Mean adhesive force ± SEM (n = 10).

[c] Mean number of discharged spirocysts ± SEM (n = 10).

[d] Intrinsic adherence of discharged spirocysts calculated from listed mean values of response parameters using Eq. 2 and a value of 32.9 mgf for S_t (Table I).

[e] Response parameters measured in ASW.

[f] Molarity of sensitizer producing half-maximal discharge of spirocysts, $K_{0.5}$.

[g] Molarity of sensitizer producing maximal discharge of spirocysts, EC_{100}.

Models of changes in intrinsic adherence of discharging mastigophores

To account for the declining hyperbolic shape of the i_m-discharge plot, we propose and test three models. Physical models such as our lateral pressure model and our capsule size model attempt to explain the shape of the i_m-discharge plot in terms of physical mechanisms. Models of fixed heterogeneity, such as our dilution/recruitment

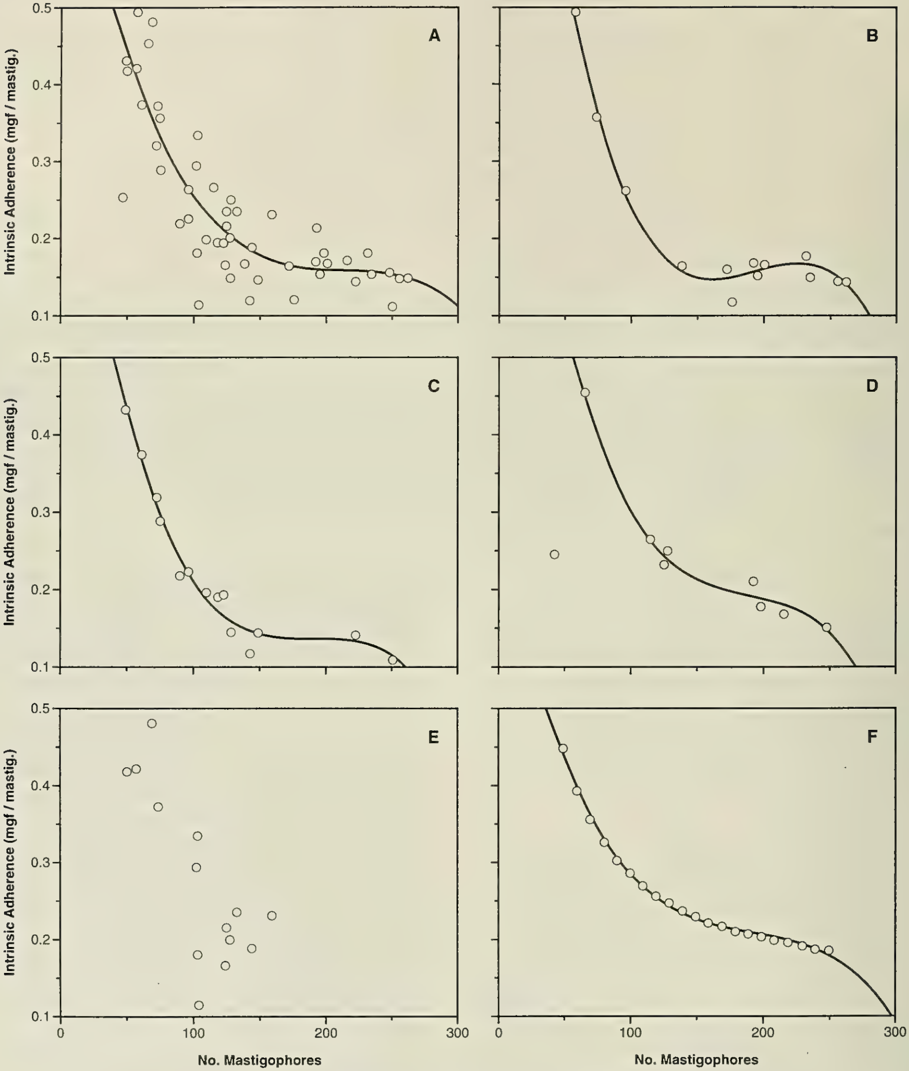

Figure 3. i_m-discharge plots of mastigophore intrinsic adherence (i_m) as a function of the number of discharged mastigophores. A. The i_m-discharge plot of i_m values calculated from Eq. 1 using data pooled from dose-responses to NANA, histamine, glycine, proline (open symbols), and ASW controls (solid symbols). Curved line is a best-fit third-degree polynomial ($R^2 = 0.71$). B. The i_m-discharge plot from dose-responses to NANA of adhesive force and numbers of discharged mastigophores and spirocysts ($R^2 = 0.99$). C. The i_m-discharge plot from dose-responses to histamine of adhesive force and numbers of discharged mastigophores

model, attempt to explain the i_m-discharge plot in terms of different mastigophore-bearing CSCCs characterized by distinct and constant i_m values.

Lateral pressure model. For our lateral pressure model, we propose that the intrinsic adherence of discharging cnidae arises in significant part from lateral pressure and 9,,,R,I0xcompressive forces generated within the ectoderm by the turgid state of adjacent undischarged cnidae. A decrease in that pressure, due to the contraction of cnidae at discharge, could cause a discharge-dependent decrease in measured i_m values in proportion to the number of discharging cnidae, such as observed in Figure 3. Indeed, we observe a 29% decrease ($P < 0.0001$) in the mean width of mastigophores after discharge; much greater than the decrease in capsule lengths (2.6%; Table III). Such changes reflect a 50% decrease in calculated volume (Thomason, 1988) during discharge of mastigophores from the tentacles of Carolina strain anemones.

The lateral pressure model is difficult to test experimentally, but, as a physical model for changes in i_m, it should apply invariantly. Validation or invalidation of this model depends upon the consistency or inconsistency of the inverse correlation of changing i_m values to the numbers of discharging mastigophores. We note several such deviations from the inverse correlation between i_m and discharge. While the i_m-discharge plots of NANA- and histamine-sensitized anemones generally show consistency of correlation (Fig. 3B, C), the plot of glycine-sensitized discharge shows one or two exceptions (Fig. 3D), and the plot of proline-sensitized discharge shows several exceptions (Fig. 3E). We conclude that while mastigophore capsules contract significantly during discharge, such changes do not cause, but may contribute to, the measured decreases in i_m values with increasing numbers of discharging mastigophores.

Capsule size model. Cnida capsules from anthozoans are prolate ellipsoidal in shape. In our capsule size model, we state that large diameter mastigophores require more force to dislodge them from the tentacle than smaller diameter mastigophores. It then follows that differences in the cross-sectional diameter of mastigophores discharged from Type B and Type C CSCCs will account for the observed differences of i_m. Thus, the capsule size model predicts that high-i_m mastigophores discharged from Type C CSCCs under control conditions will have wider capsules than mastigophores discharged from chemosensitized Type B CSCCs.

Table III

Dimensions of discharged and undischarged mastigophore capsules

Agonist	Length (μm)	Width (μm)
A. Discharged, Carolina Strain[a]		
Control	31.6 ± 0.2	5.1 ± 0.1
Glycine (5.4×10^{-12} M)[b]	30.5 ± 0.3	5.0 ± 0.1
Histamine[b]		
(9.7×10^{-10} M)	30.8 ± 0.3	5.1 ± 0.1
(10^{-7} M)	30.2 ± 0.2	5.0 ± 0.1
Avg.	30.5 ± 0.2	5.1 ± 0.1
N-acetylneuraminic Acid[b]		
(5×10^{-9} M)	32.6 ± 0.3	5.2 ± 0.1
(8×10^{-8} M)	32.8 ± 0.5	5.0 ± 0.1
(10^{-5} M)[c]	33.2 ± 0.2	5.3 ± 0.1
Avg.	32.8 ± 0.2	5.2 ± 0.1
B. Undischarged[d]		
Carolina Strain	32.4 ± 0.5	7.2 ± 0.3
Miami Strain	31.7 ± 0.5	6.0 ± 0.3

[a] Measurements represent the mean of 100 mastigophore capsules selected at random after being discharged onto 30% gelatin-coated probes by Carolina strain anemones in response to combined chemical and mechanical stimulation.

[b] Molarity of sensitizer producing half-maximal discharge of spirocysts, $K_{0.5}$.

[c] Molarity of sensitizer producing maximal discharge of both spirocysts and mastigophores, EC_{100}.

[d] Measurements represent the mean of 102 mastigophore capsules selected haphazardly from tentacle squash preparations.

We measured the dimensions of mastigophore capsules discharged onto test probes from control anemones (*i.e.*, discharged from Type C CSCCs) and from anemones chemosensitized at $K_{0.5}$ and EC_{100} levels of various sensitizers (*i.e.*, discharged from both Type B and Type C CSCCs in ratios ranging from 2:1 to 5:1, respectively). We discern no significant differences between the mean widths of discharged mastigophores from control and chemosensitized anemones (Table III). We conclude that the measured three-fold difference in i_m values cannot be accounted on the basis of differences in the sizes of discharged mastigophore capsules. We also conclude that mastigophores discharged from Type B CSCCs and from Type C CSCCs do not differ in terms of capsule size.

In addition, undischarged mastigophores from tentacle squash preparations of Carolina strain anemones used in the present study are significantly wider ($P < 0.001$) and slightly longer ($P < 0.05$; Table III) than the undischarged capsules from monoclonal Miami strain anemones used

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and spirocysts ($R^2 = 0.98$). D. The i_m-discharge plot from dose-responses to glycine of adhesive force and numbers of discharged mastigophores and spirocysts ($R^2 = 0.99$). E. The i_m-discharge plot from dose-responses to proline of adhesive force and numbers of discharged mastigophores and spirocysts. F. Simulation of dilution/recruitment model in which are plotted the weighted average i_m values calculated from Eq. 3 in which the contributions of mastigophores discharged from Type C CSCCs are assumed to be constant.

in a previous study (Geibel *et al.,* 1988). While undischarged nematocysts from the monoclonal Carolina strain measure larger than those from the Miami strain, the mean i_m value calculated for Carolina strain nematocysts of 0.18 mgf (1.76 μN; Fig. 2) is the same as that calculated for the narrower Miami strain nematocysts of 0.17 mgf (1.67 μN; Geibel *et al.,* 1988). Because differences in capsular widths between strains do not seem to give rise to significant differences in mean i_m values and because the measured differences in i_m values of nematocysts discharged from control and chemosensitized anemones cannot be accounted for on the basis of differences in capsular widths, we conclude that differences in nematocyst diameter, of the range encountered in the present study, do not significantly affect the intrinsic adherence of mastigophores.

Dilution/recruitment model. We can reproduce the general shape of the i_m-discharge plot using a model in which a constant number of high-i_m mastigophores discharged from Type C CSCCs are numerically diluted by increasing numbers of lower-i_m mastigophores discharged from Type B CSCCs. We call our model the dilution/recruitment model. Our model can be expressed in the following equation for the weighted average i_m value:

$$i_m = [(n_{mC})(i_{mC}) + (n_{mB})(i_{mB})]/n_m \qquad \text{(Equation 3)}$$

in which,

i_m is the weighted average intrinsic adherence of all discharging mastigophores;
n_{mC} is the number of mastigophores discharged from Type C CSCCs under control conditions and assumed to be constant (50 mastigophores; see Fig. 3);
i_{mC} is the intrinsic adherence of mastigophores discharged from Type C CSCCs under control conditions and assumed to be fixed at 0.45 mgf (Fig. 3);
n_{mB} is the number of mastigophores discharged from Type B CSCCs and varies (*e.g.,* from 0 to 250 mastigophores);
i_{mB} is the intrinsic adherence of mastigophores discharged from Type B CSCCs and is assumed to be fixed (*e.g.,* 0.12 mgf; Fig. 3);
n_m is the total number of mastigophores discharged (*i.e.,* $n_m = n_{mB} + n_{mC}$).

A simulation of our dilution/recruitment model (Fig. 3F), in general, shows similarities in shape to the i_m-discharge plots sensitized by NANA (Fig. 3B), by histamine (Fig. 3C), by glycine (Fig. 3D), and by proline (Fig. 3E). The dilution/recruitment model simulates the i_m-discharge plot for glycine (Fig. 3D), except for one data point corresponding to the highest tested concentration of glycine. Our model also seems to reproduce the general shape of the i_m-discharge plots for NANA (Fig. 3B) and histamine (Fig. 3C), except that figures 3B and 3C decline more steeply than the simulated plot of the dilution model and level off at lower values. Our model simulates some of the changes in the value of i_m for proline-sensitized discharge (Fig. 3E), except at high, desensitizing levels of proline where the value of i_m declines abruptly. These i_m-discharge plots suggest that the dilution/recruitment model generally, but not fully, describes the changes in i_m caused by sensitizing with NANA, histamine, and glycine.

Discussion

The properties of cnidae

Spirocysts are "adhesive organelles" (Mariscal *et al.,* 1977). By inference, spirocysts have been reputed to be the primary contributors of cnida-mediated attachment between the tentacle and inanimate substrates: ". . . spirocysts are responsible for the adhesion of tentacles to shells" (McFarlane and Shelton, 1975); and ". . . spirocysts are the primary adhesive organelle in such situations" (*i.e.,* substrate attachment) ". . . at least in *Anemonia*" (Conklin and Mariscal, 1976). Spirocysts have also been reputed to be contributors of cnida-mediated attachment to prey: (i) ". . . spirocysts . . . discharge to hold the prey" (Williams, 1968); (ii) "It is possible that the commonly observed 'stickiness' of sea anemone tentacles when lightly contacted is largely due to the discharge of spirocysts. . . . Similarly, the initial adhesion of the tentacles of a sea anemone to an unacclimated anemone fish could also be due in large part to the discharge of spirocysts (*e.g.,* Mariscal, 1970)" (Mariscal *et al.,* 1977); and (iii) "The primary function of spirocysts is in adhesion, both to substrate materials and to food objects" (Mariscal, 1984). In contrast, the everting tubule of nematocysts penetrate and envenomate prey, but also entangle (Ewer, 1947). By inference, nematocysts seem to have been relegated to playing a minor role in mediating attachment between tentacle and target (Williams, 1968; Shelton, 1980).

Several authors have attempted to determine the roles of different kinds of nematocysts in hydrozoans by observing the cnidae deployed during the capture of prey (Ewer, 1947; Purcell, 1984; Purcell and Mills, 1988). However, few functional comparisons between discharging spirocysts and nematocysts in anthozoans have been performed (Williams, 1968; McFarlane and Shelton, 1975). Attempts to ascertain cnida function based on numbers of discharged spirocysts and nematocysts retained on targets (*e.g.,* McFarlane and Shelton, 1975) tacitly, but incorrectly, assume that the tentacle adherence for each kind of cnida are equal. We recently noted that measurements of adhesive force correlate closely to the number of discharged mastigophores (Giebel *et al.,* 1988) and that the dose-responses of chemosensitized nematocyst discharge from Type B CSCCs correlate much more closely to the adhesive force dose-responses than to the dose-responses of chemosensitized spirocyst discharge (Thorington and Hessinger, 1990).

In acontiate sea anemones, cnida-mediated prey capture is regulated by both afferent and efferent mechanisms. Afferent mechanisms of cnidae influence detection and selection of prey and regulate the selective discharge of cnidae under a hierarchy of interactions: (i) between prey and predator (Thorington and Hessinger, 1988b); (ii) between CSCCs of the same and different tentacles (Hessinger *et al.,* 1992; Watson and Hessinger, 1994a); (iii) between remote sensory cells and the CSCCs (Mire-Thibodeaux and Watson, 1993); (iv) between the sensory supporting cells and the effector cnidocyte within the same CSCC (Watson and Hessinger, 1989); and (v) between chemo- and mechanoreceptor systems located on the same supporting cells (Watson and Hessinger, 1991). Efferent mechanisms of cnidae, on the other hand, determine the manner in which discharged cnidae are used to apprehend and retain captive prey. We distinguish at least three types of interaction between targets and cnida based on efferent mechanisms: (i) cnida penetration; (ii) cnida adhesion; and (iii) tentacle adherence. Until now, efferent mechanisms of discharging cnidae have not been directly studied nor measured.

Contributions to adhesive force

The measured adhesive force represents the force required to separate the test probe from the tentacle expressed in hybrid units of milligrams-force (mgf) or newtons (μN) and is an aggregate measure of both cnida-independent and cnida-mediated contributions (equation 1). In this report we have systematically measured and analyzed these contributions.

Cnida-independent contributions to adhesive force. The cnida-independent contribution to adhesive force was measured as the stickiness of the tentacle (S_t) to test probes in the absence of discharging cnidae (Table I). We inhibited the discharge of cnidae by using either cold (4°C) Ca-free ASW on living anemones or 10% formalin in ASW at room temperature. The two methods gave essentially equal measures of tentacle stickiness.

Tentacle stickiness appears to be a major contribution to the adhesive force measured with gelatin-coated targets. We infer that tentacle stickiness contributes to the retention of captured prey to the extent that the prey surface is smooth and adhesively interactive with the tentacle mucus.

Cnida-mediated contributions to adhesive force. Cnida-mediated contributions to adhesive force reflect both the number and the kind of discharging cnidae attaching to the target. The contribution of each kind of cnida to adhesive force is determined by opposing forces operating on the discharged cnida; one from the tentacle and another from the target. The force operating from the tentacle determines the extent to which the cnida capsule is attached to the tentacle and is referred to as tentacle adherence (Cormier and Hessinger, 1980). Likewise, the force operating from the target determines the extent to which the everted cnida tubule is attached to the target and is referred to as cnida adhesion. Under conditions in which cnida adhesion exceeds tentacle adherence, all discharged cnidae are retained on the target and the adhesive force measures the adherence of the cnidae to the tentacle.

Cnida adhesion. Factors affecting cnida adhesion are related to the nature of the everted tubule and to the physical and chemical characteristics of the target. As the hardness of the target is increased, progressively more mastigophores are retained until a point is reached beyond which progressively fewer discharging mastigophores attach to the target, due to the inability to penetrate (Thorington and Hessinger, 1990). For maximum adhesion of penetrant mastigophores, the surface of the target must be sufficiently soft to permit the everting tubule to penetrate the target, yet sufficiently hard to retain them when the target is withdrawn from the tentacle. Maximum retention of discharged mastigophores occurs with probes coated with 30–40% gelatin. Under such conditions mastigophore adhesion exceeds its adherence to the tentacle, and the contribution of mastigophores to adhesive force is a measure of tentacle adherence.

For maximum adhesion of discharged spirocysts, the target surface must be sufficiently hard that the sticky, everted tubules are not easily detached when the target is withdrawn from the tentacle. Coating test probes with 30% and higher concentrations of gelatin causes maximal retention of discharged spirocysts (Thorington and Hessinger, 1990). In addition, the surface of the target must be sufficiently reactive with the bioadhesive material of the everted spirocyst tubule for the everted tubules to attach firmly. Little is currently known about the chemistry of the spirocyst adhesive except that it binds N-acetylated sugars with high affinity (Thorington and Hessinger, 1990). In the present experiments, test probes have been constructed either to maximize or to minimize cnida adhesion. Specifically, 75% gelatin-coated probes maximize retention of spirocysts and minimize adhesion of the mastigophores, while 30% probes maximize the cnida adhesion of both kinds (Thorington and Hessinger, 1990).

Tentacle adherence. Several factors potentially influence tentacle adherence; some passive and some active. Passive factors potentially include considerations of the size and shape of the cnida capsules, of the lateral pressure exerted by the adjacent epithelium, as well as any possible cytoskeletal elements, such as the participation of anchoring fibrillar baskets and hemidesmosomal junctions, as occur in hydrozoan nematocytes (Cormier and Hessinger, 1980; Wood and Novak, 1982). In this paper, we show no evidence indicating that such passive factors as capsule size or lateral pressure directly influence tentacle adherence in *A. pallida* (Table III). Active factors potentially involve the contraction of intracellular cytoskeletal elements, such as has been proposed for cnidocytes of *Physalia* (Hessinger and Ford, 1988), and the constriction of adjacent accessory cells, such as the supporting cells that surround cnidocytes in anemone feeding tentacles. Our model indicates that in anemone feeding tentacles, cnidocytes function as passive effectors, triggered to discharge by contracting supporting cells. According to this model, tentacle adherence is produced by a sustained contraction of the supporting cells within the CSCCs.

In our measures of tentacle adherence, it is necessary that all of the discharged cnidae be retained on the test probe following the separation of the target from the tentacle (*i.e.*, cnida adhesion exceeds tentacle adherence). When these conditions are met, as they are when using 30–40% gelatin-coated probes (Thorington and Hessinger, 1990), and when the tentacle stickiness is known (Table I), then the measurement of adhesive force can be used to calculate tentacle adherence. In addition, when the numbers of different discharged cnidae are also known, then it is possible to calculate the contribution of individual cnidae to tentacle adherence—a unitary parameter termed the intrinsic adherence.

Intrinsic adherence. The intrinsic adherence is a limit or unitary parameter of discharged cnidae that represents the adhesive force contributed by a single discharged cnida against a target probe. The intrinsic adherence of each kind of cnida is a biologically relevant parameter that bears on its role in holding struggling prey onto the tentacle.

Contributions of spirocysts to adhesive force. Ours is the first measurement of the intrinsic adherence of either spirocysts or nematocysts. If we assume that the nematocysts retained on probes coated with 75% gelatin do not contribute to adhesive force, then the intrinsic adherence of discharged spirocysts (i_s) in *A. pallida* tentacles measures about 0.016 mgf (0.157 μN) for spirocysts from Type B CSCCs, and less than half that value for spirocysts from Type C CSCCs. However, our measures of i_s are overestimates due to our simplifying assumption that the mastigophores attached to 75% probes do not contribute to the measured adhesive force. Our assumption is not likely correct, because the attached mastigophores would not be retained if they were loosely attached. In other words, if mastigophores are retained on 75% probes, then their cnida adhesion exceeds their tentacle adherence and such mastigophores contribute to the measured adhesive force in proportion to the product of their numbers and intrinsic adherence. If we assume that the intrinsic adherence of spirocysts is insignificant and then calculate the value of i_m on 75% probes from equation 1 using the data on Table II (excluding glycine), the resulting average value of i_m is 0.19 mgf (1.86 μN). This value is consistent with the mean value of i_m in Figures 2 and 3. Thus, in our judgment, the value of i_s is negligible and spirocysts do not directly contribute to adhesive force.

Contributions of mastigophores to adhesive force. In the present study, using the Carolina strain of *A. pallida*, we verify our earlier report with the Miami strain of the same species (Geibel *et al.*, 1988) that the adhesive force measurements correlate positively with the number of discharged mastigophores (Fig. 2). From the slope of the linear correlation between the number of discharged mastigophores and the measured adhesive force, the mean i_m value is 0.18 mgf (1.76 μN) for the Carolina strain, and for the Miami strain it is 0.17 mgf (1.67 μN; Geibel *et al.*, 1988). The i_m-discharge curves (*i.e.*, i_m plotted against number of discharging nematocysts; Fig. 3) indicate that the values of i_m vary inversely and hyperbolically with the number of discharging nematocysts, ranging from a maximum value of about 0.45 mgf (or 4.41 μN)/mastigophore at low numbers of discharged mastigophores from Type C CSCCs in control anemones to 0.15 mgf (or 1.47 μN)/mastigophore at relatively high numbers of mastigophores from Type B plus Type C CSCCs in chemosensitized anemones. These values of i_m are very high in relation to the minimal or negligible values estimated for i_s. It seems reasonable that the intrinsic adherence of mastigophores should be high in relation to the values for spirocysts, given that the capsules of mastigophores are thick-walled and multi-layered (Blake *et al.*, 1988), whereas the capsules of spirocysts are thin-walled and single-layered (Mariscal *et al.*, 1976).

The declining i_m-discharge plot. We find that the observed three-fold change in i_m does not correlate with variations in capsule diameter nor is it caused by decreases in lateral pressure from the discharge of adjacent cnidae (Table III). In general, the decline in i_m with increasing numbers of discharging mastigophores is simulated by the recruitment/dilution model in which there are two populations of discharging mastigophores characterized by fixed high and low i_m values originating from Type C and Type B CSCCs, respectively. Under control (*i.e.*, non-chemosensitized) conditions and under desensitizing concentrations of chemosensitizer, a constant number of mastigophores discharged from Type Cs predominate, causing the measured i_m to be high (*i.e.*, about 0.45 mgf or 4.41 μN). With sensitizing concentrations of chemosensitizer, the discharge of progressively greater numbers of mastigophores from Type B CSCCs occurs, causing the value of i_m to decrease asymptotically to about 0.15 mgf (1.47 μN). The general fit of the dilution/recruitment model with our data also supports our designation of Type B and Type C CSCCs. The recruitment/dilution model, however, fails to account for the steepness of the decline of the i_m-discharge plot for NANA and histamine (Fig. 3B, C) and does not account for some of the i_m values plotted for proline (Fig. 3E). These deviations from the recruitment/dilution model suggest that the contribution to adhesive force of mastigophores discharging from Type C CSCCs may not be constant as we have assumed.

Roles of mastigophores and spirocysts in the capture of prey

Our findings indicate that discharging mastigophores, but not spirocysts, secure gelatin-coated targets to the feeding tentacles of *A. pallida.* From these findings, we infer that penetrable prey are secured by similar means. Our findings also indicate that the stickiness of the tentacle surface contributes significantly to the measured adhesive force. Thus, tentacle mucus likely contributes to the retention of small prey to the tentacles.

While undischarged spirocysts are about three times more numerous than mastigophores on the tentacles of *A. pallida,* the value of i_m for Type C CSCCs is at least 30 times higher than the maximum estimate of i_s. Because the value of i_s is low (Table II) to negligible in relation to the value of i_m, and because the dose-responses of discharging spirocysts do not coincide with the dose-responses of adhesive force (Thorington and Hessinger, 1990), we conclude that discharging spirocysts do not directly secure struggling prey to the tentacle. If spirocysts participate in securing prey, their action is likely indirect or restricted to very small prey. The data on Table II for 75% probes may be interpreted to suggest that spirocysts indirectly contribute to the retention of prey by assisting the attachment of everting mastigophore tubules to impenetrable surfaces, as suggested by McFarlane and Shelton (1975). We speculate that discharging spirocysts may also assist in prey capture by interfering with and fouling the swimming appendages of prey.

It is possible to estimate the minimum number of mastigophores and spirocysts needed to retain struggling prey to the tentacle, if the dynamics of swimming prey are known. The principal prey of small acontiate anemones, such as *A. pallida,* are small swimming crustaceans. The sole prey of laboratory-reared *A. pallida* used in the present study are freshly hatched *Artemia salina* nauplii (mass of approximately 0.01 mg). Based upon high-speed motion analysis of swimming *Artemia* nauplii, we calculate that they exert a maximum combined force (viscous drag plus inertial force) of approximately 0.2 μN per power stroke while swimming at an average velocity of 1.5 mm/s and a stroke frequency of 7.5 Hz (unpub.). We conclude that a single discharged mastigophore (i_m values of 4.41 to 1.47 μN) is more than sufficient to secure an individual, struggling *Artemia* nauplius to the tentacle of *A. pallida.* On the other hand, it is not conceivable that a single discharged spirocyst could tether a struggling nauplius unless it also fouls a swimming appendage. In our judgment, more than one spirocyst would be needed. For larger prey, such as *Cyclops,* with a mass of 1.025 mg, which exerts 6.6 μN of force per power stroke (Alcaraz and Strickler, 1988), at least two mastigophores from Type C CSCCs or five from Type B CSCCs, or some combination thereof, would be needed. It seems unlikely, however, that spirocysts alone would be capable of capturing and retaining such a massive prey without the involvement of mastigophores.

Conclusions

1. The ability of discharging cnidae to secure captured prey on tentacles relies upon two properties common to all cnidae: the adhesion of the cnida tubule to the prey and the adherence of the cnida capsule by the tentacle. This report provides the first measure of tentacle adherence and of the contributions of specific types of cnidae to adhesive force.

2. The upper limit of the calculated intrinsic adherence of spirocysts (i_s) is between 0.006 to 0.016 mgf (0.059 to 0.157 μN). The true value of i_s is likely much smaller to insignificant.

3. The value of i_m for a mastigophore discharged from Type C CSCCs under non-chemosensitized conditions is approximately 0.45 mgf (4.41 μN). We are not yet able to obtain accurate values of i_m for mastigophores discharged from Type B CSCCs, but a lower limit is in the range of 0.15 mgf (1.47 μN).

4. The mean value of i_m does not vary with different kinds of chemosensitizers, but varies inversely with the total number of discharging mastigophores. The dilution/recruitment model based upon the discharge of a variable number of mastigophores from low-i_m Type B CSCCs and a fixed number of mastigophores from high-i_m Type C CSCCs, in general, simulates the inverse correlation between i_m and number of discharging nematocysts.

5. The contribution of tentacle stickiness (S_t) to adhesive force is significant, suggesting that tentacle stickiness contributes, although non-selectively, to the retention of captured prey.

6. Mastigophores discharged from Type C CSCCs under control conditions are the same size as mastigophores

discharged from Type B CSCCs during chemosensitization, and differences in mean i_m values do not appear to be due to differences in the mean diameters of capsules nor to decreases in capsule volume during discharge.

7. The number of discharging mastigophores and spirocysts decreases by four-fifths and two thirds, respectively, at 4°C, and is completely blocked in Ca-free ASW.

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Acid-Base Status of the Oyster *Crassostrea virginica* in Response to Air Exposure and to Infections by *Perkinsus marinus*

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Abstract. Hemolymph acid-base variables were investigated in the Eastern oyster, *Crassostrea virginica,* to determine its responses to air exposure and to infections by the parasite *Perkinsus marinus.* Infected and uninfected oysters were subjected to two treatments of temperature (21° and 30°C) and air exposure (5 and 24 h). Upon exposure to air, oysters underwent a respiratory acidosis that remained uncompensated in uninfected oysters but was partially compensated in highly infected oysters at both 21° and 30°C. The acidosis was significantly greater in oysters with high infections. Hemolymph in uninfected oysters had a greater buffering capacity (−6.80 ± 0.76 SEM slykes) than hemolymph in highly infected oysters (−3.30 ± 0.50 SEM slykes). Calcium ion concentrations in hemolymph increase when the hemolymph becomes acidic, suggesting that shell decalcification plays a role in buffering the acid. During air exposure, although oysters do not visibly gape, they access air and are apparently not completely anaerobic.

Introduction

Crassostrea virginica (Gmelin), the Eastern (or American) oyster, occurs along the east coast of North America from the Gulf of St. Lawrence, Canada, to Key Biscayne, Florida, and along the shores of the Gulf States. In South Carolina estuaries, the species is essentially limited to the intertidal zone (Burrell *et al.,* 1984). In its habitat, *C. virginica* encounters harsh living conditions that may contribute to physiological stress and vulnerability to disease. Large fluctuations in natural environmental conditions such as temperature, salinity, and oxygen occur routinely in oyster habitats (Shumway and Koehn, 1982). Oysters may also encounter fluctuations in levels of anthropogenic pollutants including heavy metals, pesticide runoff, and hydrocarbons. In addition, oyster populations such as those in South Carolina endure air exposure twice daily due to tides. Periodically, they can also be exposed to the type of hypoxic and hypercapnic (high CO_2) conditions reported in tidal creeks in the Charleston Harbor estuary (Cochran and Burnett, 1996).

Although man has played a major role in the decline of oyster populations through over-harvesting, pollution, and the degradation of oyster habitat, other factors have contributed to oyster mortalities. Chief among them are diseases, notably MSX (*Haplosporidium nelsoni*) and "dermo" (*Perkinsus marinus*). Periodic epizootics have devastated oyster populations. It is not unusual for 75% of the oysters in a bed to have *P. marinus* disease in one summer, and infection rates are typically between 70% and 90% in a given year during the warm season (Andrews and Hewatt, 1957). The impact of *P. marinus* infections on oyster populations has been quantified by Mackin (1951), Ray (1953, 1954), and Ray and Chandler (1955). Associated with the disease are high incidences of annual mortality, usually exceeding 50% (Quick and Mackin, 1971).

Perkinsus marinus (Levine, 1978) is a protozoan pathogen of oysters (Schmidt and Roberts, 1989). Physiological abnormality accompanies the development of *Perkinsus* disease in oysters. The disease is distributed by the hemolymph to all parts of the body; in histological sections, infective spores (usually 2–20 μm in diameter) can be seen both intra- and intercellularly (Cheng, 1973). Progression of disease leads to extensive tissue damage,

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abscesses, and retarded gonadal development. Menzel and Hopkins (1955), Andrews (1961), and Paynter and Burreson (1991) have reported retarded oyster growth as well. Although the exact mode of death has yet to be elucidated, infected oysters that become severely emaciated typically gape and then die. Death is probably caused by tissue lysis and embolism of circulatory passages (Andrews and Hewatt, 1957). In general, however, sublethal manifestations of parasitism in estuarine organisms—such as that of *P. marinus* on oysters—have not been considered heretofore.

The acid-base physiology of oysters is of particular interest because changes in acid-base status can influence biochemical processes, including the deposition of shell that is essential to oyster growth (Booth *et al.,* 1984; Burnett, 1988). Exposure to air induces significant acidosis in the hemolymph of bivalves; this acidosis is generally not compensated (Booth *et al.,* 1984). Intertidal oysters experience hours of air exposure during which they have no access to water. Acid-base balance is also of interest since conditions of hypoxia and hypercapnia, which can contribute to changes in acid-base variables, have been observed in the Charleston Harbor estuary (Cochran and Burnett, 1996), which supports a large oyster population. The present study examines the acid-base status of hemolymph in *C. virginica:* how it is influenced by air exposure and temperature and how it responds to infections of *P. marinus.*

Materials and Methods

Two populations of *Crassostrea virginica* were examined in this study. Local South Carolina oysters from the Charleston Harbor estuary were used as experimental animals. Uninfected oysters from the Chesapeake Bay in Maryland were used as controls because so few (2 of the 200 animals examined) uninfected oysters were found in the study population.

Collection and preparation of oysters

Experimental animals were from a bed in the Folly River, Folly Beach, South Carolina (32°38.8′ N; 79°57.1′ W), where the salinity is normally greater than 25 ppt. Oysters were collected from the low- and mid-intertidal zone at low tide. Specimens had a mean shell height of 85.8 mm (SEM = 0.8) and were about 2–3 years old. All epibionts were removed in order to obtain individual oysters for physiological measurements. The oysters were scrubbed clean, placed in aquaria in well-aerated 25 ppt seawater at the Grice Marine Biological Laboratory, and allowed 24 h to recover before being prepared for experiments.

Oysters uninfected with *Perkinsus* served as controls and were collected from the Wye River in the upper Chesapeake Bay where they were tray-cultured in low-salinity water (8–10 ppt) by Chesapeake Mariculture, Queenstown, Maryland. The animals were packed in ice and shipped overnight to the Grice Marine Laboratory. There they were placed in aquaria with recirculating water and acclimated to 25 ppt salinity in a stepwise fashion. Over the course of the experiments, control oysters had a mean shell height of 60.4 mm (SEM = 0.6). They were certified free of *Perkinsus* disease by Dr. Kennedy Paynter of the University of Maryland. In addition, all oysters were assayed for *Perkinsus* in our laboratory.

Oysters were prepared for hemolymph sampling by drilling a 1-mm hole in the shell over the adductor muscle and covering the hole with dental dam cemented into place with cyanoacrylate glue. The oysters were then returned to holding tanks and allowed 48 h to recover. Oysters from Maryland were held in aquaria separate from those housing oysters collected in South Carolina.

Bioassay for Perkinsus marinus

All physiological measurements were made on oysters in the holding conditions described, after which they were immediately assayed for the presence and intensity of *Perkinsus* infections. As soon as possible after measuring total CO_2 and pH, the oysters were sacrificed and necropsies performed to assay for *P. marinus.* The fluid thioglycollate medium (FTM) technique, being specific for *P. marinus* (Ray, 1952; Mackin and Ray, 1966) was used, as modified by Quick (1972), for culturing the parasite. The rectum was excised from the oyster and placed in FTM culture medium prepared according to Howard and Smith (1983). After 7 days the tissue explant was analyzed for parasite hypnospores according to procedures by Howard and Smith (1983).

The intensity of the parasitic infection was determined according to the index developed by Ray (1953, 1954, 1966) and modified by Quick and Mackin (1971), where 0 = no hypnospores in the entire sample and 6 is ≥ 1001 hypnospores per 5-mm field. Oysters were placed into one of three infection categories: none (index = 0), low (index = 1, 2, or 3), or high (index = 4, 5, or 6).

Experimental design and statistical analysis

Most experiments were designed for a multiway three-factor analysis of variance (ANOVA) to differentiate among the effects of temperature, air exposure, and infection status on pH and total CO_2. The physiological responses of hemolymph pH and total CO_2 (defined as all forms of CO_2 including molecular CO_2, HCO_3^-, and $CO_3^=$) in control and experimental oysters were measured against three treatments: temperature (21° and 30°C), air exposure (immersion longer than 24 h compared with 5-h and 24-h emersion), and infection level ("None" compared with "High" levels) under controlled laboratory conditions. In each of the treatments salinity was maintained at 25 ppt. All aquaria were well aerated and the oysters were not fed before or during the experiments. Water pH was always at or above 8.0.

The length of air exposure in oyster habitats depends upon a regular sinusoidal variation in tidal height, amplitude changes associated with spring and neap tides, and other factors (McMahon, 1988). A 5-h emersion is a typical maximum air exposure for local oysters during periods of spring tides. Because high-intertidal oysters are commonly exposed for even longer periods, and oysters have been known to tolerate days of air exposure, 24 h was chosen as the upper limit of exposure.

Most experiments were conducted over the period of July through November when *in situ* water temperatures were similar to those employed in the study (21° and 30°C). Furthermore, July, August, September, and October are the months when *P. marinus* infection levels are at their peak, coinciding with the warmest water temperatures.

Values are expressed throughout as mean ± 1 standard error of the mean (SEM). Total CO_2 and pH values were tested for variance according to Sokal and Rohlf (1981) by a three-factor ANOVA. Due to nonuniform variances in total CO_2 (heteroskedasticity), these values were natural log transformed before analysis. Statistically significant interactions were further analyzed by graphing techniques. The significance of differences for pH and total CO_2 between uninfected and highly infected oysters was determined by a Mann-Whitney rank sum test.

Differences in calcium ion concentrations between air exposure treatments within an infection group were tested with a Mann-Whitney rank sum test (uninfected group) or a Kruskal-Wallis one-way analysis of variance on ranks (low-infection and high-infection groups) (Table I). Differences in mean hemolymph pH, total CO_2, and calcium ion concentrations between oysters emersed in air and oysters emersed in nitrogen were tested using a student's *t* test or, when a test for normality failed, a Mann-Whitney rank sum test (Table II).

Hemolymph acid-base status

At the start of each experiment about 80 μl of hemolymph was anaerobically drawn, using a 1-ml glass syringe and a 23-gauge needle, from the sinus of the adductor muscle of each oyster. The samples were placed on ice until measurements were made. In all experiments, hemolymph was drawn just once from each animal.

Total CO_2 was measured by the method of Cameron (1971) or with a Capni-Con 5 total CO_2 analyzer. The pH of the oyster hemolymph was measured using a capillary pH electrode (Radiometer BMS2 Mk2) calibrated at experimental temperatures with precision Radiometer buffers.

In separate experiments to test the hypothesis that calcium ions are mobilized from the shell in response to acidification, calcium ion concentration was measured in hemolymph from oysters held at 21°C and 25 ppt salinity. Hemolymph was sampled as described above, and the concentration of free calcium ions was measured with a calcium ion electrode (Radiometer).

Because our results indicated a significant increase in hemolymph P_{CO_2} upon air exposure, we wanted to test the hypothesis that oysters were somehow accessing oxygen in air and using it metabolically to produce CO_2 even though their valves were assumed to be tightly closed. Thus, we performed additional experiments in which we compared acid-base variables (measured as above) for two groups of oysters—one emersed in an atmosphere of pure nitrogen for 24 h and the other emersed in air.

Buffering capacity of the hemolymph

Buffering properties of the oyster hemolymph were determined at 21° and 30°C by incubating hemolymph samples of highly infected oysters and uninfected oysters ($n = 4$ for each) in thermostatted tonometers (Radiometer BMS2 Mk 2) at four different CO_2 pressures (2, 4, 15, and 22 torr) provided by Wösthoff precision gas mixing pumps. Hemolymph was drawn as described above from each oyster and centrifuged for 2 min in a microcentrifuge. Hemolymph (80 μl) was incubated in a thermostatted tonometer for at least 30 min at a particular P_{CO_2}. Total CO_2 and pH were then measured for each sample as above, permitting the calculation of p*K*. Appropriate p*K* (calculated from oyster hemolymph) and CO_2 solubility constants (Truchot, 1976) for the experimental temperatures were used to create pH–bicarbonate diagrams.

The buffering capacity of each hemolymph sample was determined from the slope of the linear relationship between pH and bicarbonate ion concentrations at the different CO_2 pressures. The resulting slope is a measure of the non-bicarbonate buffers ($\Delta[HCO_3^-]/\Delta pH$ = slykes) in the hemolymph.

Results

Hemolymph acid-base status and buffering capacity

Values for hemolymph pH and total CO_2 were analyzed using pH–bicarbonate diagrams (Figs. 1 and 2). At 21°C, highly infected oysters have a pH significantly ($P = 0.0003$) different from those of uninfected oysters at 0 h. Both the highly infected and the uninfected oysters underwent a respiratory acidosis induced by air exposure (Fig. 1). The acidosis at 24 h was slightly greater for the uninfected Maryland oysters (pH = 6.607) than for the highly infected South Carolina oysters (pH = 6.653). The uninfected oysters showed no significant compensation after 24 h. The

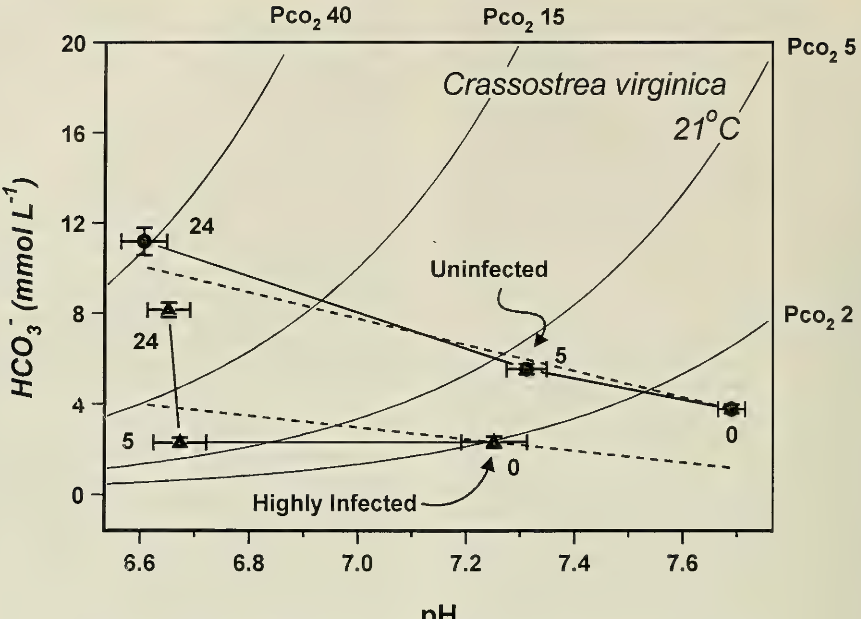

Figure 1. A pH–HCO_3^- diagram showing the acid-base status of oyster hemolymph at 0, 5, and 24 h of air exposure at 21°C. P_{CO_2} isopleths (curved lines) are given in torr. *In vitro* buffer lines are shown as dashed lines. Circles represent oysters uninfected with *Perkinsus marinus;* triangles represent oysters with "high" infections. Values are mean ± SEM; *n* for each experiment ranged from 22 to 56.

uninfected group had a higher P_{CO_2} (~42 torr) than did the highly infected group (~28 torr). The acidosis in the highly infected group was uncompensated at 5 h of air exposure, but was partially compensated at 24 h.

At 30°C, highly infected oysters had a pH significantly ($P = 0.0004$) different from that of uninfected oysters at 0 h. Both groups underwent a respiratory acidosis induced by air exposure (Fig. 2). The acidosis at 24 h was greater for the uninfected oysters (pH 6.73) than for the highly infected group (pH 6.94). The uninfected oysters showed no significant compensation after 24 h of air exposure. The uninfected group had a P_{CO_2} similar to that at 21°C (~44 torr) and greater than that of the highly infected group (~31 torr). The acidosis in the highly infected group was uncompensated after 5 h of air exposure, but was partially compensated at 24 h. The change in hemolymph pH with temperature in uninfected oysters ($\Delta pH/°C = 0.045$) was twice that predicted for passive temperature effects on pH and commonly reported for ectotherms.

Calcium ion concentration in hemolymph of uninfected oysters was lower than that in hemolymph of oysters with low or high infections ($P < 0.05$; Kruskal-Wallis one-way analysis of variance on ranks; Table I). Exposure of oysters to air resulted in an elevation of hemolymph calcium ion concentrations in all treatment groups (Table I).

Oysters emersed and exposed to nitrogen for 24 h had an acidosis level similar to that of air-exposed oysters regardless of infection level, but total CO_2 (with one exception) and calcium ion concentrations differed significantly between treatments (Table II).

The buffering properties of the hemolymph were examined by comparing the slopes of the *in vitro* buffer lines (Figs. 1 and 2). At 21°C, the slope for the *in vitro* buffer line (indicating the non-bicarbonate buffering strength of the hemolymph) was −5.56 slykes for uninfected oysters ($\Delta[HCO_3^-]/\Delta pH$) and −2.57 for highly infected oysters. At 30°C, the slope of the *in vitro* buffer line was −7.81 slykes for uninfected oysters and −4.02 for highly infected oysters. The slopes of the individual *in vitro* buffer lines for highly infected and uninfected oysters at the two temperatures were compared with *t* tests. At 21°C, the slopes were not significantly different ($P = 0.082$; df = 3).

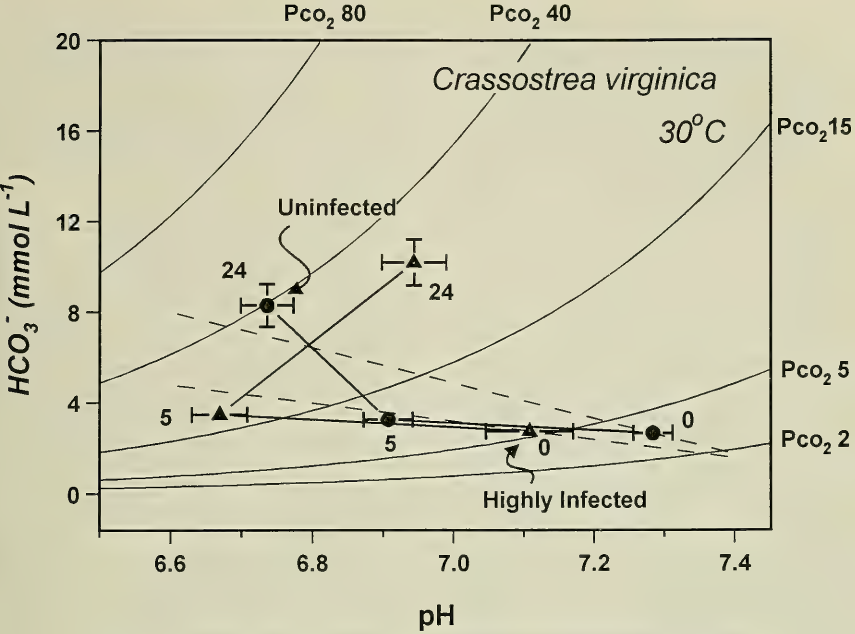

Figure 2. A pH–HCO_3^- diagram showing the acid-base status of oyster hemolymph at 0, 5, and 24 h of air exposure at 30°C. P_{CO_2} isopleths (curved lines) are given in torr. *In vitro* buffer lines are shown as dashed lines. Circles represent oysters uninfected with *Perkinsus marinus;* triangles represent oysters with "high" infections. Values are mean ± SEM; *n* for each experiment ranged from 22 to 56.

At 30°C, however, the slopes of the lines were significantly different ($P = 0.025$; df = 5).

Infection levels

Mean *P. marinus* infection levels of oysters collected in South Carolina in 1993 peaked in September ($\bar{x} = 3.6 \pm 0.2$). The mean level was 1.8 ± 0.1 in July 1993 and 2.0 ± 0.2 in November 1993 (Fig. 3). Throughout the study period, bioassays for the detection of *P. marinus* were performed on more than 200 South Carolina oysters. Only two oysters were found to be infection-free; thus the infection prevalence was 99%. Among all treatments, the infection level for the control Maryland oysters was zero. In fact, throughout the study, in no case was infection detected in the Maryland oysters ($n = 159$).

Discussion

The use of oysters from two different populations (Maryland and South Carolina), while not ideal, was necessary because no uninfected oysters were available from South Carolina. Nevertheless, we feel that the physiological effects we observed in this study were induced by infections of *P. marinus* and not by differences between the oysters from different sources. Most of the results presented here were obtained from uninfected (Maryland) oysters and highly infected (South Carolina) oysters. South Carolina oysters with infection intensities intermediate between these two extremes (*i.e.,* oysters with "low" infections) show intermediate responses. For example, the results in Table I suggest that intermediate (low) infections of *P. marinus* yield a result intermediate between uninfected and highly infected oysters. Furthermore, a similar pattern of intermediate responses with "low" infection intensities was found among the acid-base variables (*e.g.,* Figs. 1 and 2), but we have not presented the data. We suggest that the intermediate responses are a function of infection intensity and that the uninfected Maryland oysters fit into the pattern. We cannot, however, exclude the possibility that Maryland oysters have a different acid-base physiology from South Carolina oysters.

When the valves of an oyster are closed, the P_{CO_2} rises and the pH of the hemolymph declines (Crenshaw and

Table I

Hemolymph calcium ion concentration (mmol/l) as a function of air exposure in oysters uninfected with Perkinsus marinus, *oysters with low infections, and oysters with high infections at 21°C and 25 ppt salinity; statistical tests are between immersed oysters and air exposed oysters within an infection group*

Treatment		Uninfected	Low infection (Index = 1–3)	High infection (Index = 4–6)
Immersed	Mean	3.9	5.4	6.4
	SEM	0.14	0.27	0.36
	n	60	41	21
Air exposed (5 h)	Mean		9.1	9.1
	SEM		0.27	0.45
	n		34	15
	p		<0.05	<0.05
Air exposed (24 h)	Mean	10.6	11.3	13.9
	SEM	0.87	1.00	0.80
	n	5	20	26
	p	<0.0001	<0.05	<0.05
	Statistical test	Mann-Whitney rank sum	Kruskal-Wallis one-way analysis of variance on ranks	

Neff, 1969; Booth *et al.*, 1984). Some intertidal bivalves can sustain prolonged exposures to anoxia and display impressive anaerobic capacities (Grieshaber *et al.*, 1994). McMahon (1988) suggested that during valve closure many intertidal bivalves remain almost completely anaerobic to minimize evaporative water loss. Several authors (*e.g.*, Dugal, 1939; Crenshaw and Neff, 1969) have shown that the molluscan shell helps to buffer decreases in pH produced by anaerobic metabolism. It is not known, however, whether bivalves possess other mechanisms to regulate the pH of their body fluids.

Booth *et al.* (1984) observed respiratory acidosis in the mussel *Mytilus edulis* when it was exposed to air. The authors attributed the acidosis entirely to observed increases in P_{CO_2}. Although they presumed that anaerobic metabolism was occurring during air exposure, they concluded, on the basis of work by Zurburg *et al.* (1982), that no metabolic acids entered the hemolymph. Partial compensation for the acidosis was observed and attributed to shell decalcification because an increase of calcium ions was measured in the hemolymph. An increase in ammonia levels was also implicated in compensation.

The present study reveals that oysters exposed to air develop a respiratory acidosis of the hemolymph that is comparable to the pattern observed by Booth *et al.* (1984) for mussels, although more severe. These results are also similar to the acid-base responses of other intertidal invertebrates (Truchot, 1988; Grieshaber *et al.*, 1994). The primary source of the respiratory acidosis in *C. virginica* during air exposure is most likely the retention of CO_2 produced metabolically. The oysters kept their valves closed during exposure to air, when CO_2 accumulated. CO_2 is produced by aerobic pathways; however, as the limited O_2 stores within the shell become depleted, it is possible that CO_2 is produced by anaerobic means (reviewed by de Zwaan and Wijsman, 1976). On the other hand, our observations on emersed oysters exposed to an atmosphere of nitrogen indicate that oysters are not fully isolated from the aerial environment and, thus, not fully anaerobic.

It is likely that oysters, like other bivalves, lower their metabolism when they are exposed to air and that a portion of their metabolism is aerobic (Widdows and Shick, 1985). The acidosis produced in a nitrogen atmosphere was just as severe as that produced in air (Table II), but the amount of total CO_2 present in the hemolymph was generally less. In addition, the calculated P_{CO_2} of hemolymph was much lower ($\sim$20 torr) in nitrogen-exposed oysters than in air-exposed oysters ($\sim$42 torr). These results indicate that metabolic acids, possibly acetic acid, propionic acid, or succinic acid, are produced in the nitrogen atmosphere. Less CO_2 is also produced. Thus, acidosis has an obvious "metabolic" component in the absence of oxygen.

The acid-base balance in many aquatic animals is thought to depend mainly upon active transport of ions between the extracellular fluid and the environment. Both Lindinger *et al.* (1984) and Booth *et al.* (1984) demonstrated that *M. edulis* appears to buffer H^+ loads by passive dissolution of carbonates, thereby producing CO_2. Their findings were based in part upon increases in hemolymph Ca^{++} concentration; a result consistent with ours. Interestingly, the hemolymph calcium concentrations in nitrogen-exposed oysters are lower than those in air-exposed oysters (Table II). These results are in contrast to what is expected. Crenshaw and Neff (1969) demonstrated a direct relationship between the appearance of succinic acid and calcium ions in the extrapallial fluid of *Mercenaria mercenaria.* Because we sampled hemolymph, there may be a decrease in the transport of calcium between the hemolymph and the extrapallial fluid under completely anaerobic conditions. Dugal and Fortier (1941) also reported that the calcium concentration in the mantle cavities of oysters increased from 40 to 400 mg/100 ml (10 to 100 mmol/l) during air exposure, accompanied by an increase in CO_2 content. Here again, there may be a difference between changes in calcium ion concentrations in the hemolymph and other fluid compartments.

The acidosis quantified in the present study is of concern for several reasons. The data suggest that, to buffer the acid, calcium ions are released through dissolution of the $CaCO_3$ bound in the shell. The net result of shell dissolution may be retardation of growth. The effects of severe hemolymph acidosis, as observed here, are even more far-reaching. At these hemolymph pH levels, intracellular

Table II

Hemolymph acid-base variables in oysters emersed in air or a nitrogen atmosphere at 21°C and 25 ppt salinity and with infections of Perkinsus marinus; *statistical tests are between oysters emersed in air and oysters emersed in nitrogen within an infection group*

Infection level	Treatment		Total CO_2 (mmol/l)	pH	Ca^{++} (mmol/l)
Uninfected	Emersed 24 h in air	Mean	11.5	6.58	10.6
		SEM	1.00	0.095	0.87
		n	5	5	5
		Test	*t* test	*t* test	*t* test
		p-value	<0.0001	0.928	0.0073
	Emersed 24 h in N_2	Mean	4.9	6.57	7.5
		SEM	0.45	0.054	0.53
		n	10	10	10
Low infection	Emersed 24 h in air	Mean	11.9	6.58	11.25
		SEM	0.99	0.044	0.99
		n	20	16	20
		Test	Mann-Whitney rank sum test	*t* test	*t* test
		p value	0.0062	0.710	0.041
	Emersed 24 h in N_2	Mean	5.4	6.55	7.5
		SEM	0.47	0.061	0.63
		n	7	7	7
High infection	Emersed 24 h in air	Mean	9.5	6.77	13.9
		SEM	0.78	0.051	0.79
		n	26	24	26
		Test	*t* test	*t* test	Mann-Whitney rank sum test
		p value	0.0519	0.608	0.0192
	Emersed 24 h in N_2	Mean	5.9	6.83	8.35
		SEM	0.25	0.124	0.77
		n	6	6	6

pH will be low as well (Lindinger *et al.*, 1984; Walsh *et al.*, 1984). The resulting systemic acidosis may also affect cellular metabolism by decreasing the rate of glycolysis or by shifting the use of one metabolic pathway to another (see Grieshaber *et al.*, 1994, for review).

Acid-base balance in *C. virginica* is clearly affected by infections with *P. marinus*. Most protozoan parasites are ammonotelic and excrete most of their nitrogen as ammonia. Ordinarily, ammonia readily diffuses across cell membranes into the surrounding medium. This avenue may not be available to oysters during air exposure and the resultant valve closure (Schmidt and Roberts, 1989). Ammonia is also a common waste product of oysters and bivalves in general. It is possible that NH_3 contributes to the pH compensation in infected oysters by buffering H^+. CO_2, lactate, pyruvate, and short-chain fatty acids also are common waste products of these protozoans (Schmidt and Roberts, 1989). *P. marinus* is undoubtedly a source of CO_2 that accounts for a portion of the respiratory component of the acidosis.

The pH–bicarbonate diagrams (Figs. 1 and 2) show that the highly infected oysters, unlike those uninfected, compensated partially for the acidosis after 24 h of air exposure. Compensation was greater at the higher temperature (30°C). We do not know why the control (Maryland) oysters did not compensate for their respiratory acidosis. The age and size of these oysters may provide a clue. The Maryland oysters were somewhat younger and smaller

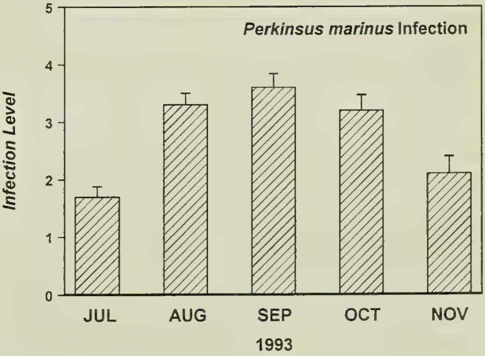

Figure 3. Bar graph depicting *Perkinsus marinus* infection levels in South Carolina oysters in the months of July through November 1993 from the Folly River collection site. Values are mean ± SEM.

than the South Carolina oysters, so there may be some adaptive value in retaining shell for growth rather than allowing dissolution of calcium carbonate to create a buffer for H^+ ions. Another possibility is that the biochemical matrix of young oyster shell is different from the shell of older oysters.

In the present study, the pattern of infection intensity (Fig. 3) peaked during warm-weather months when water temperatures are about 30°C, the experimental temperature treatment that yielded the largest acidosis. Similar findings were obtained by Burrell *et al.* (1984). All of the above findings lead us to speculate that a positive feedback mechanism may operate in conditions of high temperature and acidity that favor the propagation and development of *P. marinus* infection. The parasite may in turn contribute to the acidic environment of its host tissues and extracellular fluids, further perpetuating itself. Interestingly, in experiments where *P. marinus* was cultured, Chu and Greene (1989) observed significant but unexplained drops in the pH levels of the cultures. It is likely that the low pH was due to the metabolic production of CO_2.

This investigation into the physiological effects of *Perkinsus marinus* on oyster physiology has shown that under conditions of air exposure and high temperature, oysters with high *Perkinsus* infections undergo a severe acidosis. The acidosis is due to elevated hemolymph CO_2 and is partially compensated in infected oysters, but uncompensated in uninfected oysters. Many infected oysters remained at or below a pH of 6.7 during air exposure. Profound negative effects may result from this acidosis since pH affects, and may even regulate, physiological and biochemical processes (Grieshaber *et al.,* 1994).

Perkinsus marinus may thrive under the acidic conditions it induces in oysters. Whether environmental conditions that induce an acidosis in oysters (*e.g.,* elevated environmental CO_2 and air exposure) stimulate *P. marinus* infections is unknown but currently under investigation. Certainly, *P. marinus* is a significant problem in South Carolina waters. Infection intensities are high in warm-water months (Fig. 3; Crosby and Roberts, 1990), and prevalence may be as high as 100%.

This study also points out the need to consider the possibility and effects of parasitism in research involving an organism's physiology and biochemistry. Oysters infected with *Perkinsus* are not obviously different from those that are uninfected; thus, researchers must look for infections. Oysters that are infected and acidotic may respond to environmental challenges differently from oysters that are not infected and not acidotic. Anderson (1975) suggested that grass shrimp parasitized by epicaridean isopods have lowered rates of oxygen uptake, and that this response may reflect changes in the lipid metabolism of the host. It is not possible to generalize about the physiological and metabolic responses marine organisms have to parasitism, but it should be recognized that a parasite can have profound effects.

Acknowledgments

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Stimulants of Feeding Behavior in Fish: Analyses of Tissues of Diverse Marine Organisms

WILLIAM E. S. CARR, JAMES C. NETHERTON III, RICHARD A. GLEESON,
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Abstract. Analyses of the free amino acids, quaternary amines, guanido compounds, nucleotides, nucleosides, and organic acids in extracts of tissues from 10 species of marine teleost fishes and 20 species of invertebrates are reported. With multidimensional scaling techniques, the relative concentrations of the above chemicals in fishes, molluscs, and crustaceans are shown to cluster into separate taxon-specific groups. The greatest differences are between the fishes and the two groups of invertebrates. Similarities are more evident between the molluscs and crustaceans where eight of the nine most abundant substances are identical: *i.e.*, betaine, taurine, trimethylamine oxide, glycine, alanine, proline, homarine, and arginine. The major tissue components in the fishes and invertebrates are correlated with compounds previously shown to stimulate feeding behavior in 35 species of fish. Glycine and alanine are major tissue components and are also the two most frequently cited feeding stimulants in the 35 species. Molluscs and crustaceans each contain high concentrations of five of the most frequently cited stimulants (glycine, alanine, proline, arginine, and betaine); these substances all occur in much lower concentrations in fish. Some minor tissue components, such as tryptophan, phenylalanine, aspartic acid, valine, and uridine 5′-monophosphate, are, however, important feeding stimulants for some fish species. Stimulants for herbivores and carnivores are often different. Several major feeding stimulants are substances that serve as "compensatory solutes," stabilizing enzymes and structural proteins.

Introduction

Many aquatic animals have acute chemical senses. Thus, the occurrence of sufficient concentrations of specific substances may elicit pronounced behavioral responses. The effects of chemicals on the following behaviors of aquatic animals have been reviewed: feeding (Carr and Derby, 1986a), feeding deterrence (Bakus *et al.*, 1986), predator avoidance (Elliot *et al.*, 1989), larval hatching (Rittschof *et al.*, 1989), habitat recognition by larvae (Morse, 1990), gamete development and spawning (Stacey *et al.*, 1994), and homing (Stabell, 1992). Regarding fish, Hara (1993) recently provided a thorough review of the structure of the chemosensory system and the facets of behavior affected by chemicals.

Many studies show that four types of common, low-molecular-weight metabolites, acting either alone or as components of mixtures, serve as potent attractants or as stimulants of feeding behavior. These substances are free amino acids, quaternary ammonium compounds, nucleotides or nucleosides, and organic acids (*e.g.*, Carr and Derby, 1986a; Zimmer-Faust *et al.*, 1988; Jones, 1992). Other, less frequently cited, substances evoking feeding behavior are the opines arcamine and strombine (Sangster *et al.*, 1975), lecithin (Harada, 1987), and the plant product dimethyl-β-propiothetin (Nakajima *et al.*, 1989).

The identification of natural feeding stimulants and stimulant mixtures has provided information for basic studies of receptor types (*e.g.*, Caprio *et al.*, 1993; Hara, 1994), mixture interactions (Carr and Chaney, 1976; Derby and Ache, 1984a; Carr and Derby, 1986a, b), neural coding (Derby and Ache, 1984b), transduction mechanisms (Brand and Bruch, 1992), and perireceptor events (Carr *et al.*, 1989). Further, the recognition that feeding behavior can be evoked by specific chemicals has been important in efforts to produce artificial baits for fishing

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activities (Carr, 1984; Løkkeborg, 1990; Jones, 1992), and to increase the palatability of rations used in aquaculture (Mackie and Mitchell, 1985; Takeda and Takii, 1992).

This report provides analyses of extracts of 30 species of fishes and invertebrates used as bait. The compositions of fish, mollusc, and crustacean extracts are compared. The relative abundances of major tissue components are correlated with their roles as behavioral feeding stimulants in 35 species of fish. The roles of minor tissue components, and the differences between stimulants for carnivores and herbivores, are also discussed.

Materials and Methods

Species analyzed

Table I lists the 17 species of marine organisms that we analyzed at the Whitney Laboratory (St. Augustine, Florida), as well as 13 species analyzed elsewhere by others. Most of the species are used as bait, but several are also used for human consumption.

The methods of extraction and analyses described in the following two sections are those used by us at the Whitney Laboratory. The procedures used by others are described in the references cited in Table I.

Preparation of extracts

The following tissues were analyzed: muscle from fishes, crustaceans, and an octopus; mantle from squid; and all soft parts from oyster and razor clam. Extracts were prepared from a pool of at least four animals to obtain representative samples; but with octopus, only a single large specimen was available. Fresh tissue was homogenized in a blender for 2 min in cold deionized water (4:1 or 3:1; volume:weight). Each homogenate was centrifuged at 5°C at 5800 × *g*. The supernatant was ultrafiltered consecutively at 5°C in Amicon Centripep-10 tubes (10,000 MW cutoff), then Amicon Centricon-3 tubes (3,000 MW cutoff). Each final ultrafiltrate, containing only substances of low molecular weight, was divided into aliquots and stored at −85°C until analyzed.

Analytical procedures

Amino acids were analyzed by two procedures to increase the number of compounds measured. In Procedure 1, ninhydrin reagent was used with a Hitachi amino acid analyzer (Model 835). In Procedure 2, amino acids were derivatized with *ortho*-phthaldialdehyde (Lindroth and Mopper, 1979) and separated by HPLC on an octadecylsilane column with 4-μm spherical particles (Waters Nova-Pak C_{18}, 3.9 × 150 mm). Elution at 1.4 ml/min was with a propionic acid buffer system (Turnell and Cooper, 1982; Pastoris *et al.*, 1988): buffer A consisted of water, 0.25 *M* aqueous propionic acid in 0.35 *M* Na_2HPO_4 (pH 6.5) and acetonitrile (82:10:8); buffer B consisted of water, 0.25 *M* aqueous propionic acid in 0.35 *M* Na_2HPO_4 (pH 4.0), acetonitrile, methanol, and dimethylsulfoxide (32:10:30:25:3). We used a step gradient modified from Pastoris *et al.* (1988), such that at 3, 15, 30, 40, and 50 min after sample injection, the percentage of buffer B was

Table I

List of species analyzed

No.	Common name	Scientific name	Source and condition of specimens[a]
		Fish	
1.	Black mullet*	*Mugil cephalus*	local (live)
2.	Hake, Pacific*	*Merluccius productus*	Seattle (frozen)
3.	Herring, Atlantic*	*Clupea harengus*	Seattle (frozen)
4.	Herring, Pacific*	*C. harengus pallasi*	Seattle (frozen)
5.	Mackerel, Atl.*	*Scomber scombrus*	Norway (frozen)
6.	Mackerel, chub**	*Scomber japonicus*	
7.	Mackerel, horse**	*Trachurus japonicus*	
8.	Pink salmon*	*Oncorhynchus gorbuscha*	Seattle (frozen)
9.	Red sea bream**	*Chrysophrys major*	
10.	Spanish sardine*	*Sardinella anchovia*	local (frozen)
		Crustaceans	
11.	Blue crab 1*	*Callinectes sapidus*	local (live)
12.	Blue crab 2**	*Portunus trituberculatus*	
13.	Horsehair crab**	*Erimacrus isenbeckii*	
14.	King crab**	*Paralithodes camtschaticus*	
15.	Shrimp, northern*	*Pandalus borealus*	Norway (frozen)
16.	Shrimp, Pacific**	*Penaeus japonicus*	
17.	Shrimp, pink*	*Penaeus duorarum*	local (live)
18.	Spiny lobster**	*Panulirus japonicus*	
19.	Krill 1*	*Euphausia superba*	Norway (frozen)
20.	Krill 2**	*Meganyctiphanes norvegica*	
21.	Krill 3**	*Thysanoessa inermis*	
		Molluscs	
22.	Blue mussel**	*Mytilus edulis*	
23.	Eastern oyster*	*Crassostrea virginica*	local (live)
24.	Razor clam, Pac.*	*Siliqua patula*	Seattle (live)
25.	Shortneck clam**	*Tapes japonica*	
26.	Octopus*	*Octopus dofleini*	Seattle (frozen)
27.	Squid 1*	*Ilex argentius*	Norway (frozen)
28.	Squid 2*	*Ilex illezibrosis*	Seattle (frozen)
29.	Squid 3*	*Ilex sp.*	local (frozen)
30.	Squid 4**	*Loligo vulgaris*	

[a] Source and condition only for specimens analyzed at Whitney Laboratory.

* Analysis done at the Whitney Laboratory, St. Augustine, FL. Specimens from Norway were provided by the Institute of Fishery Technology Research in Bergen. Specimens from Seattle were provided by Marco Marine Seattle, WA. Frozen specimens were frozen immediately after capture, shipped in dry ice, and maintained at −85°C until analyzed. Analyses of species 1, 11, 17, and 23 were published previously (Carr and Derby, 1986b).

** Species analyzed at other laboratories. References by species number are as follows: 6, 7, 9, 12, 13, 14, 16 and 18—Konosu and Yamaguchi (1982); 20 and 21—Saether and Mohr (1987); 22—Macke *et al.*, (1980); 25—Hidaka (1982); 30—Mackie (1973).

increased from 0 to 13, 25, 45, 80, and 100, respectively. Fluorescent derivatives were detected with a Bio-Rad Model 1700 fluorometer fitted with a 360-nm excitation filter and a 440-nm emission filter; peaks were integrated with a Waters Model 730 data module. Compounds were identified and quantified by comparison with standards.

Nucleosides were analyzed by HPLC with a reverse-phase Brownlee Spheri-5 RP-18 column, 4.6 × 100 mm. Buffer A was 0.02 M KH_2PO_4, pH 5.6; buffer B was 60% methanol: 40% water (Assenza and Brown, 1980). Compounds were eluted at 1.0 ml/min with a gradient of 0% to 45% buffer B in 15 min. Nucleosides were detected by UV absorption at 260 nm and were identified and quantified by comparison with standards.

Nucleotides were analyzed by HPLC with an ion-exchange column (Whatman Partisil 10 SAX), 4.6 × 250 mm. Buffer A was 0.007 M KH_2PO_4, 0.007 M KCl, pH 4.0; buffer B was 0.25 M KH_2PO_4, 0.5 M KCL, pH 5.0 (modified from McKeag and Brown, 1978). Elution at 3.0 ml/min was isocratic at first with 100% buffer A for 3 min. This was followed by a linear gradient from 0% to 100% buffer B for 30 min. Nucleotides were detected by UV absorption at 254 nm and identified and quantified by comparison with standards.

Quaternary ammonium and guanido compounds were analyzed on thin layer cellulose plates developed with butanol:acetic acid:water (6:1.5:2.5). Dragendorff reagent (Bregoff *et al.,* 1953) was used to visualize quaternary amines, whereas Sakaguchi reagent (Dubnoff, 1957) was used for guanido compounds, particularly octopine and creatine. Quantification was by a visual comparison of the minimum detectable amounts of standard and extracted compounds.

Organic acids were analyzed by HPLC with a Bio-Rad Aminex HPX-87H organic acid column, 7.8 × 300 mm, preceded by a Bio-Rad cation-exchange guard column. Separation was obtained by isocratic elution with 0.01 N H_2SO_4 at a flow rate of 0.4 ml/min (modified from Guerrant *et al.,* 1982). Organic acids were detected by UV absorption at 210 nm, then identified and quantified by comparison with standards.

Statistical comparison of composition of extracts from different species

The concentrations of low-molecular-weight components in the species examined were further analyzed with multivariate statistical techniques to determine whether the overall chemical compositions of species in the same taxon (*e.g.,* crustaceans, molluscs, and teleost fish) are more similar to each other than to those of species from different taxa.

Multidimensional scaling (MDS) was selected to represent our highly complex data set in a form that is easy to interpret but that still explains most of the variance in the data (*e.g.,* Schiffman *et al.,* 1981; Bieber and Smith, 1986; Di Lorenzo, 1989). MDS places the variables (in our case, species) in a spatial map such that distances between variables indicate the degree of similarity between them. Hence variables that are distant have relatively different properties, whereas variables that are close are more similar.

MDS first measures the similarity between each pair of variables; *i.e.,* the similarity in the chemical compositions of each pair of species. This yields a matrix of similarity measurements that represent the entire data set. Because we were most interested in differences in the relative concentrations of components in different extracts, two similarity measures using relative differences were employed (Statistica software, StatSoft Inc.): the Pearson product-moment correlation coefficient (Bieber and Smith, 1986), and the *theta* value. *Theta* values are derived from vector space analysis based on linear algebra, and have advantages over Pearson correlation coefficients when values are low (Di Lorenzo, 1989).

Creation of a matrix of similarity measurements requires a value (= concentration) for each chemical for each species used in the analysis. Because the entire group of 51 chemicals was not measured in any of the 30 reported species, MDS analyses were restricted to a portion of the data: 29 chemicals in the 17 species analyzed at the Whitney Laboratory (these species are indicated by a single asterisk in Table I).

The Low-Molecular-Weight Components of Tissue Extracts

Table II presents the concentrations (mmol/kg wet weight) of 51 low-molecular-weight substances—free amino acids, quaternary amines, nucleosides, nucleotides, organic acids, and guanido compounds—in 10 species of fish and 20 species of invertebrates.

The cumulative concentrations of the substances described above (determined from the last column in Table II) are quite similar in the crustaceans (mean ± SEM = 286.5 ± 22.3 mM/kg) and molluscs (262.9 ± 39.5 mM/kg). Among the molluscs analyzed, the mean concentration in pelecypods is 285.6 ± 88.5 mM/kg, whereas in cephalopods it is 245.6 ± 27.5 mM/kg. In crustaceans, the concentrations in decapods and euphausids (krill) are 310.1 ± 18.4, and 223.5 mM/kg ± 56.3, respectively. In contrast, the mean concentration in fishes, 119.8 ± 13.5 mM/kg, is only 43%–48% of that measured in either invertebrate group.

Because exhaustive extraction procedures were not employed to prepare all extracts, the total concentrations of components may in some cases be slightly less than normal; but the relative concentrations of individual components should be representative and are the major focus of the analyses that follow.

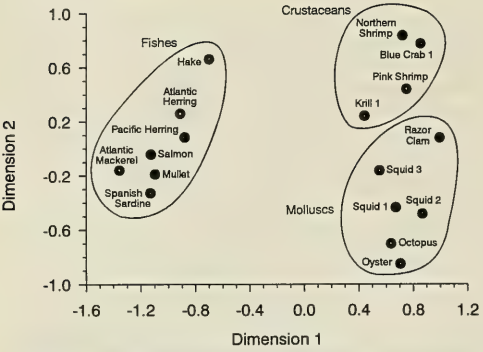

Figure 1. Degrees of similarity in the composition of 29 chemicals in extracts from 17 species of fishes, crustaceans, and molluscs as revealed by multidimensional scaling analyses using *theta* values (see text). Common names of species are as in Table II. To include Spanish sardine and black mullet in the analyses, two values were estimated: for Spanish sardine, Gln was estimated to be 0.723 m*M*/kg (= the mean value for 7 teleost species in which Gln was measured); for black mullet, Cre was estimated at 40 m*M*/kg (= the mean value for the other 9 teleost species). A value of zero was used for those chemicals indicated as "not detected" (=ND) or "unreported" (=U) in Table II.

Statistical Comparisons of Extract Compositions

Multidimensional scaling (MDS) analyses were used to discern relationships or patterns in the chemical composition of diverse species (Fig. 1). These analyses included 29 components (Ala, Arg, Asn, Asp, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Orn, Phe, Pro, Ser, Tau, Thr, Tyr, Val, Bet, Cre, Hom, TMO, AMP, ADP, ATP, GMP, IMP) from the 17 species analyzed at the Whitney Laboratory.

Figure 1 shows that species belonging to the same taxonomic group are closely clustered. Thus, teleost fish, crustaceans, and molluscs each forms a distinct group. This result shows again that the chemical compositions of the fish species are more similar to each other than they are to the compositions of either the crustacean or molluscan species. Among the invertebrates, however, two species are as close to members of the other taxon as to their own. Note that "krill 1" (Order Euphausiacea) is as close to two of the molluscs as it is to the other crustaceans (Order Decapoda). Likewise, razor clam is as close to two crustaceans as it is to the other molluscs.

Within each major taxon, species vary in the similarity of their compositions (see Fig. 1). For example, among the teleosts, the congeneric species Atlantic herring and Pacific herring are more similar to each other than they are to any other species, and black mullet, salmon, and Spanish sardine are relatively similar to each other. Hake is a relative outlier in the teleost group, although it remains clearly separate from any of the invertebrates. Within the molluscs, the three congeneric species of squid cluster closely, as do octopus and oyster, and all are relatively dissimilar to razor clam. In the crustaceans, blue crab 1 and northern shrimp are the most similar to each other and relatively dissimilar to krill 1.

Separate MDS analyses were performed on the matrix of Pearson correlation coefficients and on that of *theta* values. Because the results of these two MDS analyses were virtually identical, only the results using *theta* values are shown in Figure 1. A two-dimensional solution was sufficient to represent this data set, as indicated by scree analysis of stress values (Schiffman *et al.,* 1981; Bieber and Smith, 1986).

Major Extract Components in Different Taxa

The 10 chemical components occurring at highest concentrations (Figs. 2–5) in the extracts of a particular taxon are designated as "major extract components" of that taxon. When the components of teleosts, crustaceans, and molluscs (from Table II) are plotted in rank order (Figs. 2–5) these major extract components are revealed to be highly characteristic of the taxa.

In fish, creatine, lactic acid, and trimethylamine oxide (TMO) are the three major components (Fig. 2); each appears in all fish extracts in which an effort was made to measure them (Table II). Of these three components,

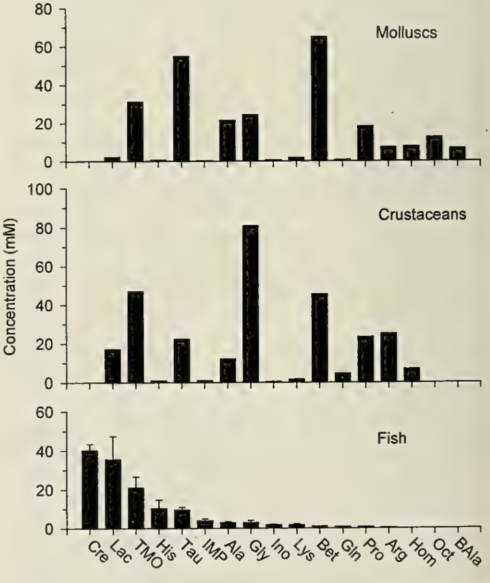

Figure 2. Mean concentrations (±SEM) and ranking of the 10 major components in fish extracts compared with those in crustaceans and molluscs. Source of data and abbreviations of substances as in Table II.

Foldout for Table II

able II

alyses of low molecular weight constituents of fish, crustaceans and molluscs.[a],[b] Concentrations = mM/kg wet weight

Common name	Genus/species	Amino acids																												Quaternary amines and guanido compounds						Nucleotides and related compounds							Organic acids								Others		Total conc. mM/kg
		ALA	βALA	AABA	GABA	ARG	ASN	ASP	CYS	GLU	GLN	GLY	HIS	1MHIS	3MHIS	HYP	ILE	LEU	LYS	MET	ORN	PHE	PRO	SER	TAU	THR	TRY	TYR	VAL	BET	CRE	CREN	HOM	OCT	TMO	AMP	ADP	ATP	GMP	IMP	HYX	INO	ACE	FUM	LAC	OXAL	PROP	PYRO	PYR	SUC	ANS	SAR	
Fish																																																					
1 Black mullet	*Mugil cephalus*	2.64	0.02	0.03		0.28	0.34	0.34		0.42	0.51	3.05	6.72		0.12	0.06	0.14	0.26	1.18	0.07	0.27	0.08	0.47	0.49	11.4	0.48		0.04	0.28	6.2			ND		5.9	0.02	0.01	ND	0.06	4.13	0.07	1			34.3								81.38
2 Hake, Pacific	*Merluccius productus*	1.33	0.17			0.514	0.008	0.059		0.357	0.033	0.809	0.056				0.116	0.277	0.434	0.03	0.089	0.074	0.334	0.246	2.55	0.198	0.087	0.093	0.579	ND	30		ND		60	0.108	0.128	0.072	0.053	2.84	0.548	1.35			7.68								111.222
3 Herring, Atlantic	*Clupea harengus*	6.18				0.104	0.016	0.098		0.644	ND	12.1	3.44				0.192	0.321	0.842	0.307	0.111	0.218	0.585	1.16	9.12	0.789	ND	0.102	0.595	ND	60		ND		40	3.81	ND	ND	ND	ND	0.765	1.58			32.8								175.879
4 Herring, Pacific	*C. harengus pallasi*	3.03				0.214	ND	0.245		1.64	4.15	3.74	0.493				0.308	0.467	2.52	0.315	ND	0.262	0.444	0.574	13.3	0.862	ND	0.146	0.678	ND	40		ND		25	ND	ND	ND	ND	ND	2.27	2.76			1.06								104.478
5 Mackerel, Atlantic	*Scomber scombrus*	0.72				0.072	0.04	ND	0.12	0.66	ND	0.528	10.5				0.09	0.16	0.72	0.116	0.028	ND	0.116	0.136	5.66	0.252	ND	0.084	0.216	ND	40		ND		4	0.003	ND	ND	ND	2.05													66.271
6* Mackerel, chub	*Scomber japonicus*	2.92	ND	ND	ND	0.631		ND		1.22		0.932	43.6	ND	ND	ND	0.534	1.07	5.09	0.134	0.297	0.242	2.26	0.571	6.71	0.924	ND	0.386	1.37		31.9	1.15			12.3	0.192	0.08	0.112		5.44		2.39	1.5		75.9		1.48		1.14	1.27			203.745
7* Mackerel, horse	*Trachurus japonicus*	2.36	ND	0.097	ND	0.172	ND	0.075		0.884	ND	1.33	18.6	ND	ND	ND	0.076	0.381	2.96	0.067	0.297	0.061	0.52	0.285	6	1.26	ND	0.055	0.512		32.7	0.973			24.3	0.082	0.199	0.225		5.25		0.746									0.108		100.575
8 Pink salmon	*Oncorhynchus gorbuscha*	4.41		0.097		0.209	0.21	0.172		1.5	0.365	2.24	2.44		1.11		0.327	0.575	0.252	0.362	0.077	0.315	0.481	0.844	12.2	0.942	0.151	0.522	0.814	ND	40		ND		9	0.046	0.025	0.03	0.031	0.756	1.74	3.29		0.077	61.2								146.81
9* Red sea bream	*Chrysophrys major*	1.46	ND		ND	0.115				0.34		1.6	0.258	ND	ND	ND	0.229	0.305	0.602			0.121	0.174	0.285	11	0.252	ND	0.11	0.256		48.1	1.5			23.2	0.274	0.12	0.179		6.6		0.559									0.075		97.714
10 Spanish sardine	*Sardinella anchovia*	4.39				0.67	ND	0.688	0.504	2.15		1.77	17.1				1.34	1.22	1.2	0.865	0.48	0.419	1.54	1.12	15.9	1.29	ND	0.625	1.36	ND	40		ND		5	ND	ND	ND	0.172	10.5													110.303
Crustaceans																																																					
11 Blue crab 1	*Callinectes sapidus*	12.8	ND			13.7	1.17	0.22	0.72	1.7	17.7	68	0.83				0.64	1.47	1.36	2.35	ND	0.56	33.4	1.59	11.3	2.42	0.79	0.61	1.91	22.2	U		2.81		20.3	0.15	1.09	5.27	ND	0.95	0.2	0.41			26.1					2.14			256.86
12* Blue crab 2	*Portunus trituberculatus*	16.2	ND	0.387	ND	18.9		1.13		2.92		59.1	1.48	ND	0.414		2.21	4.5	2.41	3.28	ND	1.21	21.8	2.19	17.1	2.02	0.294	1.27	4.1	55.2	U				5.85	1.73	0.04	ND		0.04	0.367	0.522											226.664
13* Horsehair crab	*Erimacrus isenbeckii*	17.7	ND			45.1		0.751		1.5		119	0.709				0.381	0.686	0.548	0.871		0.424	33.3	2.19	44	1.01		0.552	1.2	60.7	U				13	0.274	1.5	4.78															350.176
14* King crab	*Paralithodes camtschaticus*	20.9	0.56	0.291	0.291	44.5		0.751		4.89		81.4	2	0.059			2.36	4.8	3.07	2.55	0.178	3.27	43.6	1.62	29.7	2.94		3.09	4.61	35.6	U					1.26	0.06			0.019	0.294				14.4					0.339			309.402
15 Shrimp, northern	*Pandalus borealis*	14.7				11.8	0.385	ND	0.624	4.59	ND	93.7	0.869				1.61	2.23	1.77	1.17	2.61	ND	28.8	2.19	12.6	3.69	ND	0.67	2.88	22.5	U		10		40	0.02	0.295	1.05	ND	1.65	0.484	0.818		0.04	11.4								275.145
16* Shrimp, Pacific	*Penaeus japonicus*	4.83				51.8		ND	ND	2.31		162	1.03			ND	0.686	0.991	2.85	0.804		0.424	17.6	12.7	12	1.09	ND	1.1	1.45	64.2	U				15.5								0.83		14.4	0.333	0.945	0.62	0.795	0.51			371.798
17 Shrimp, pink	*Penaeus duorarum*	21.2	ND			8.82	1	0.83		1.65	6.3	93.7	0.32				1.06	1.9	0.49	1.04		0.54	11.4	1.34	33	0.64	ND	0.92	2.3	50.4	U		6.9		54	4.88	1.43	0.4	ND	2	0.23	0.06			27.5								336.25
18* Spiny lobster	*Panulirus japonicus*	4.71				38.7		ND	ND	0.476		143	0.838			ND	1.3	0.915	1.15	1.14		0.363	10.1	10.2	5.44	0.504	ND	0.607	1.62	82.1	U				19.2								0.67		25.7	0.333	0.54	1.47	0.795	2.29			354.161
19 Krill 1	*Euphausia superba*	4.68	3.51			8.54	0.089	0.626		0.64	0.093	12.4	0.64				0.483	0.489	1.14	0.468	ND	0.115	6.25	0.393	9.73	0.583	ND	0.428	0.915	17.5	ND		7.5		30	0.883	ND	ND	ND	2.14	ND	0.219			0.545								110.999
20* Krill 2	*Meganyctiphanes norvegica*	4.8				17.7	0.45	1.43		2.9	2.33	25.9	0.43				0.65	0.59	0.88	0.43		0.46	24.8	1.25	33.5	0.44		1.2	0.81		U				142.5	1.35	0.65	1.25		0.68		0.63										5.48	273.49
21* Krill 3	*Thysanoessa inermis*	10.8				14.5	0.65	1.6		0.73	0.93	30.2	0.58				1.35	1.84	3.5	0.33	1.8	1.28	25	1.4	36.8	0.98		0.15	2.28		U				130	0.78	0.4	0.58		0.55		0.68										16.2	285.89
Molluscs																																																					
22* Blue mussel	*Mytilus edulis*	22.8				5.34		4.73	0.416	14.3		21.3	1.8				1.53	2.52	6.85	2.68		1.21	4.78	5.71	91.1	5.46		1.66	5.55	158	U		3.46							0.42		2.52											364.136
23 Eastern oyster	*Crassostrea virginica*	7.32	1.72			0.62	0.23	0.98	0.13	1.01	0.77	4.79	0.1			0.25	0.04	0.08	0.33	0.02	0.19	0.01	2.45	0.67	33.1	0.11		0.05	0.08	20.3	U		1.99	ND	ND	0.59	0.05	ND	0.08	0.11	ND	0.07			0.64					2.14			81.02
24 Razor clam, Pacific	*Siliqua patula*	92.8	21.5	0.489		33.9	0.286	12.7		15.7	0.711	81.3	0.826				1.04	1.12	2.76	1.46	0.533	ND	8.35	5.05	111	6.42	0.307	0.79	2.8	35.5	ND		23.7	ND	14.8	2.63	ND	0.064	0.12	0.072	0.431	0.546		0.216	ND					5.34			485.261
25* Shortneck clam	*Tapes japonica*	14.5				5.37		1.57	0.19	6.99		43.7	0.56				0.79	1.53	1.71	0.7		1.18	1.42	2.25	53.1	1.05	ND	0.9	1.23	58	U		4.59			0.13	0.14			1.04	0.75	1.09		0.3						2.99			207.77
26 Octopus	*Octopus dofleini*	2.17	16.3			2.45	0.012	0.767		1.88	0.105	0.446	0.249				0.284	0.337	0.6	0.507	0.975	0.216	1.96	0.465	40.8	0.91		0.366	0.693	35	ND		ND	30	13	0.144	ND	ND	ND	0.127	1.71	ND			1.1					ND			153.573
27 Squid 1	*Illex argentinus*	12.4	ND			2.53	0.14	ND	0.7	0.685	1.22	3.32	0.725				0.41	0.725	0.515	0.57	0.225	ND	17.9	0.85	65.6	1.83		0.225	1.05	90	ND		12.5	12.5	55	1.11	0.007	ND	ND	0.215	ND	ND			5.33					ND			288.282
28 Squid 2	*Illex illecebrosus*	8.68	ND			5.2	0.097	0.107		9.34	1.25	3.3	0.745				0.521	0.862	0.74	1.06	0.372	0.647	31.7	1.16	41.4	1.8		1.01	1.26	125	ND		ND	20	50	0.575	ND	ND	ND	0.275	5.15	0.321			0.74					ND			313.312
29 Squid 3	*Illex sp.*	16.6	ND			3.59	ND	1.77	1.1	3.88	ND	5.68	0.492				1.28	2.36	1.95	1.47	1.95	2.57	37.4	2.03	46	2.3		1.1	2.15	35	U		20		50	0.058	ND	ND	0.196	0.204	ND	ND											241.13
30* Squid 4	*Loligo vulgaris*	14.2				6.03		0.631		1.66		54.8	0.457				0.991	2.02	0.734	1.1		0.787	58.7	1.47	12.4	1.73		0.579	1.47	27.3	U		1.94		35.6	0.507					1.6	0.44			4.66								231.806

[a] Abbreviations for substances: Ala = alanine; βala = β-alanine; AABA = α-amino butyric acid; GABA = γ-aminobutyric acid; Arg = arginine; Asn = asparagine; Asp = aspartic acid; Cys = cysteine; Glu = glutamic acid; Gln = glutamine; Gly = glycine; His = histidine; 1Mhis = 1-methylhistidine; 3Mhis = 3-methylhistidine; Hyp = hydroxyproline; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; Orn = ornithine; Phe = phenylalanine; Pro = proline; Ser = serine; Tau = taurine; Thr = threonine; Try = tryptophan; Tyr = tyrosine; Val = valine; Bet = betaine; Cre = creatine; Cren = creatinine; Hom = homarine; Oct = octopine; TMO = trimethylamine oxide; AMP, ADP and ATP = adenosine 5′-monophosphate, -diphosphate, and -triphosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; Hyx = hypoxanthine; Ino = inosine; Ace = acetic acid; Fum = fumaric acid; Lac = lactic acid; Oxal = oxaloacetic acid; Prop = propionic acid; Pyro = pyroglutamic acid; Pyr = pyruvic acid; Suc = succinic acid; Ans = anserine; Sar = sarcosine.

[b] ND = not detected; U = unreported in crustaceans and molluscs (*e.g.*, Prosser, 1973, pp. 740–742).

* Species analyzed at other laboratories; see Table I

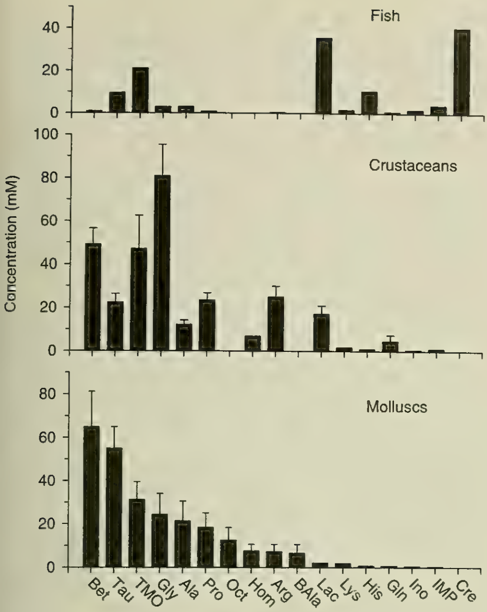

Figure 3. Mean concentrations (±SEM) and sequential ranking of the 10 major components in mollusc extracts compared with those in crustaceans and fish. Data and abbreviations as described in Figure 2 and Table II.

only TMO appears among the top five substances in the invertebrate extracts. Creatine was the dominant component in six fish species; lactic acid was dominant in three species, and TMO was dominant in a single species, the Pacific hake (*Merluccius productus*). In two species, the Atlantic mackerel (*Scomber scombrus*) and Spanish sardine (*Sardinella anchovia*), the three major components are creatine followed by histidine and taurine. Of all the analyzed substances, only creatine is confined to fish. Further, only fish have histidine, inosine 5′-monophosphate (IMP), inosine, and lysine among their 10 major components.

Extracts of molluscs and crustaceans show several clear similarities (Fig. 3). In both groups, eight of the same substances are represented among the top nine components; *i.e.,* betaine, taurine, TMO, glycine, alanine, proline, homarine, and arginine. Further, both molluscs and crustaceans have betaine, TMO, and glycine among their top four components. Notable differences include the limitation to molluscs of octopine, and other opines not measured in the current study (*e.g.,* Gäde 1980; 1988). Also, betaine is the major single component in molluscs when the analyses of both pelecypods and cephalopods are combined. Likewise, glycine is the major single component in crustaceans when the analyses of both decapods and euphausids are combined.

Among the pelecypod molluscs, taurine and betaine are the dominant components in three of the four species (Fig. 4 and Table II). In the Pacific razor clam (*Siliqua patula*), however, taurine is the dominant substance followed by glycine, alanine, and then betaine. Even greater variability exists among the cephalopod molluscs. Betaine and taurine are the two dominant components in the octopus (*Octopus dofleini*) and in one species of squid (*Illex argentinus*). In the remaining three squid species, the two dominant components are betaine and TMO in *I. illecebrosus* and *I. sp.,* and proline and glycine in *Loligo vulgaris.*

Glycine is the dominant component in extracts of all eight species of decapod crustaceans (Fig. 5 and Table II). In addition, four substances (glycine, betaine, arginine, and proline) are among the top six components in all of the species except the pink shrimp (*Penaeus duorarum*). Even the pink shrimp has glycine, betaine, and proline among its top six components.

The three euphausid crustaceans show marked similarities in composition (Fig. 5 and Table II). TMO is the dominant substance in each species. Also, excluding be-

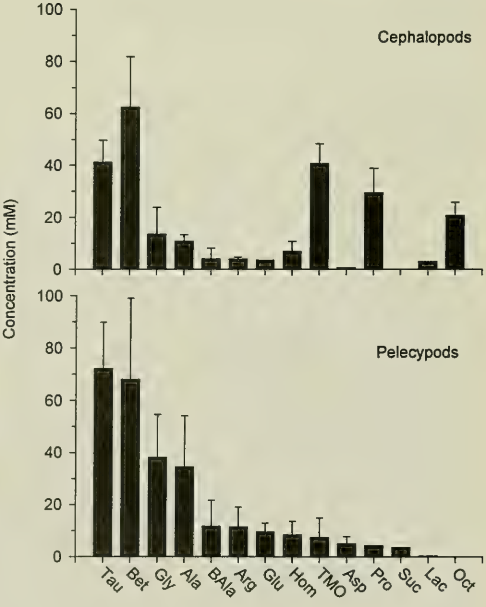

Figure 4. Mean concentrations (±SEM) and sequential ranking of the 10 major components in pelecypod and cephalopod extracts. Data source and abbreviations as described in Table II.

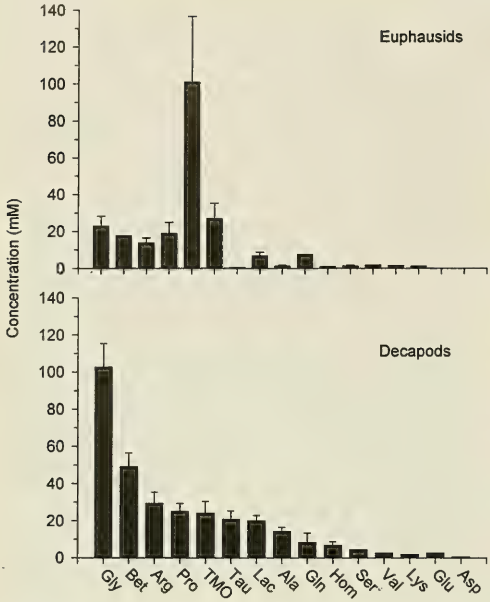

Figure 5. Mean concentrations (±SEM) and sequential ranking of the 10 major components in decapod and euphausid extracts. Data source and abbreviations as in Table II.

taine and homarine, which were measured in only a single species, the five major components in each extract are TMO, taurine, glycine, proline, and arginine.

One analysis of a decapod, the northern shrimp (*Pandalus borealis*), was omitted from Table II because the extract was made from boiled shrimp (Ellingsen and Døving, 1986; Mearns *et al.*, 1987), and we have only presented analyses of fresh tissues. Boiled shrimp are used in Norwegian fisheries, but when compared to extracts of fresh northern shrimp (Table II), those of boiled shrimp showed proportional reductions in both the total solute content and the concentrations of major components: *i.e.*, total solute concentration (53%), betaine (33%), glycine (33%), proline (39%), and alanine (45%). However, levels of arginine, TMO, and taurine remained quite similar in the fresh and boiled shrimp.

Correlation between Major Extract Components and Feeding Stimulants

The pooled set of 10 major components from fish, molluscs, and crustaceans (see Figs. 2, 3) comprises 17 substances (Table III). Thirty-five species of fish have exhibited feeding behavior to two or more of those substances either when the substances are tested individually or as part of a mixture.

Glycine and alanine are the two most frequently cited feeding stimulants, being reported, respectively, in 28 (80%) and 26 (74%) of the 35 species (see bottom of Table III). Further, **both** glycine and alanine are stimulants in 22 species. This finding, plus the close structural similarities between these 2- and 3-carbon neutral amino acids, suggest that in some cases they may activate similar or identical receptor types. Activation of similar receptors by glycine and alanine is supported by electrophysiological studies employing cross-adaptation regimes of taste receptors in both freshwater and marine catfish (Wegert and Caprio, 1991; Michel *et al.*, 1993).

In addition to glycine and alanine, four other substances, proline (37%), arginine (37%), betaine (34%), and histidine (23%) are feeding stimulants in greater than 20% of the species cited in Table III. Three of the major extract components—homarine, octopine, and creatine—are not cited as feeding stimulants in any species of which we are aware.

Interesting relationships between taxa emerge when the six most frequently cited feeding stimulants shown in Table III (*i.e.*, glycine, alanine, proline, arginine, betaine, and histidine) are compared with the extract compositions provided in Table II and Figures 2 through 5. Each of the above six feeding stimulants are referred to hereafter as a "m̲ost f̲requently c̲ited s̲timulant" (=MFCS).

Histidine is the only MFCS represented among the six major extract components of teleost fish (see Fig. 2). This amino acid is implicated in intracellular pH regulation in fish (Van Waarde, 1988), and does not occur as a major component in any of the invertebrate extracts. Other MFCSs occur at very low concentrations in the fish extracts. Creatine, the dominant component in fish extracts, has never been reported as a feeding stimulant, although it acts as a feeding deterrent in the jack mackerel, *Trachurus japonicus* (Ikeda *et al.*, 1988a).

Not only the MFCSs, but indeed most low-molecular-weight compounds, occur at considerably lower concentrations in the fish extracts than in the invertebrate extracts examined here (Table II). However only *marine* teleosts were studied, so the characteristic reduced concentration is consistent with the hyposmotic regulation of solutes occurring in these organisms (*e.g.*, Evans, 1993).

In molluscan extracts, four MFCSs—betaine, glycine, alanine, and proline—are represented among the top six extract components. Crustacean extracts also have four MFCSs—glycine, betaine, arginine, and proline—among their top six components. The popularity of molluscs and crustaceans as baits and feed additives (*e.g.*, Mackie, 1982; Franco *et al.*, 1991; Jones, 1992), and as seafoods for human consumption (Konosu and Yamaguchi, 1982), is very likely related to their high concentrations of the most frequently cited feeding stimulants.

The six "most frequently cited stimulants" described above, plus the remainder of the 17 major extract com-

Table III

Stimulation of feeding behavior in fish by the 10 major components analyzed in extracts of fish, molluscs, and crustaceans

Fish species exhibiting feeding behavior to defined substances	Major components in extracts* †														Hom Oct Cre
	Gly	Ala	Pro	Arg	Bet	His	Tau	Lys	Gln	IMP	Ino	TMO	βAla	Lac	
1. Arctic charr	M	M							M						
2. Atlantic salmon															
fry	S	S	S	S											
yearlings	M	M	M	M		M	M								
3. Brown trout fry	S	S	S	S		S									
4. Carp	M	M													
5. Catfish, bullhead	M	M													
6. Catfish, channel		S	S	S											
7. Cod	SM	SM	SM	SM										M	
8. Dab	M	M	M	M			M								
9. Dover sole 1															
Juvenile	M	M			M										
Sub-adult					S										
10. Dover sole 2															
Juvenile	M				M						M				
11. Eel, European	M	M			M										
12. Eel, Japanese															
Juvenile	M	M	M		SM	M									
Adult	SM	SM		SM			M								
13. Flounder, winter	S	S													
14. Goby	S	S											S		
15. Herring larvae	S	S	S												
16. Jack mackerel	M	M	M	M		M	M	M		SM					
17. Killifish	S	S				S									
18. Marbled rockfish		SM	SM							S	SM				
19. Oriental weatherfish	S	SM		SM		SM		S							
20. Pigfish	M				SM				M						
21. Pinfish	M				SM				M						
22. Plaice	M	M	M	M	M		M					M		M	
23. Puffer	M	M	M		M										
24. Rainbow trout	M	M	SM	SM		M	SM	M							
25. Red sea bream	SM	SM		S	M			S	S						
26. Sea robin	S		S												
27. Shark, lemon	S				S							S			
28. Shark, nurse	S				S							S			
29. Silversides		S											S		
30. Tilapia		S						S			S				
31. Turbot										S	S				
32. Walleye				S	S										
33. Whiting	SM	SM													
34. Yellowtail	S			SM		SM		S	SM	S					
35. Zebrafish		S													
Total number of species exhibiting feeding behavior	28	26	13	13	12	8	6	6	5	4	4	3	2	1	0
Percent of above species exhibiting feeding behavior	80	74	37	37	34	23	17	17	14	11	11	9	6	3	0

* Component stimulatory as a single substance (S); as part of a mixture (M); or both (SM). A blank space indicates the substance was inactive or not tested.

† Abbreviations: Gly = glycine; Ala = alanine; Pro = proline; Arg = arginine; Bet = betaine; His = histidine; Tau = taurine; Lys = lysine; Gln = glutamine; IMP = inosine-5′monophosphate; Ino = inosine; TMO = trimethylamine oxide; βAla = βalanine; Lac = lactic acid; Hom = homarine; Oct = octopine; Cre = creatine.

References by species number: 1. *Salvelinus alpinus* (Olsén *et al.*, 1986). 2. *Salmo salar* fry (Mearns, 1989); yearlings (Mearn, 1985; Mearns *et al.*, 1987). 3. *Salmo trutta* (Mearns, 1986). 4. *Cyprinus carpio* (Saglio *et al.*, 1990). 5. *Ictalurus nebulosus* (Johnsen and Teeter, 1980). 6. *Ictalurus punctatus* (Valentincic and Caprio, 1994). 7. *Gadus morhua* (Pawson, 1977; Ellingsen and Døving, 1986; Johnstone and Mackie, 1990; Franco *et al.*, 1991). 8. *Limanda limanda* (Mackie, 1982). 9. *Solea solea* juvenile (Mackie *et al.*, 1980); sub-adults (Mackie and Mitchell, 1982). 10. *Solea vulgaris* (Metailler *et al.*, 1983). 11. *Anguilla anguilla* (Mackie and Mitchell, 1983). 12. *Anguilla japonica* juvenile (Takeda *et al.*, 1984); adult (Hashimoto *et al.*, 1968; Konosu *et al.*, 1968). 13. *Pseudopleuronectes americanus* (Sutterlin, 1975). 14. *Gobiosoma bosci* (Hoese and Hoese, 1967). 15. *Clupea harengus* (Dempsey, 1978). 16. *Trachurus japonicus* (Ikeda *et al.*, 1988a, b; Takeda and Takii, 1992). 17. *Fundulus heteroclitus* (Sutterlin, 1975). 18. *Sebasticus marmoratus* (Takaoka *et al.*, 1990). 19. *Misgurnus anguillicaudatus* (Harada, 1985b; Harada *et al.*, 1987). 20. *Orthopristis chrysopterus* (Carr, 1976; Carr *et al.*, 1977). 21. *Lagodon rhomboides* (Carr and Chaney, 1976). 22. *Pleuronectes platessa* (Mackie, 1982). 23. *Fugu pardalis* (Ohsugi *et al.*, 1978; Hidaka, 1982). 24. *Salmo gairdneri* (Adron and Mackie, 1978; Jones, 1989). 25. *Chrysophrys major* (Goh and Tamura, 1980; Fuke *et al.*, 1981; Shimizu *et al.*, 1990). 26. *Prionotus carolinus* (Bardach and Case, 1965). 27. *Negaprion brevirostris* (Hodgson and Mathewson, 1978). 28. *Ginglymostoma cirratum* (Hodgson and Mathewson, 1978). 29. *Menidia menidia* (Sutterlin, 1975). 30. *Tilapia zillii* (Johnsen and Adams, 1986). 31. *Scophthalmus maximus* (Mackie and Adron, 1978). 32. *Stezostedion vitreum* (Rottiers and Lemm, 1985). 33. *Merlangius merlangus* (Pawson, 1977). 34. *Seriola guingueradiata* (Harada, 1985a; Harada *et al.*, 1987; Takeda and Takii, 1992). 35. *Brachydanio rerio* (Steele *et al.*, 1990).

ponents listed in Table III, are not the only known feeding stimulants. Some substances present in very low concentrations in food or baits are either major stimulants of fish species or, as is more often the case, make significant contributions to the activity of mixtures. In the jack mackerel, *T. japonicus,* tryptophan, a minor constituent of krill extract, was the only one of 20 amino acids in the extract that was a feeding stimulant (Ikeda *et al.,* 1988b). The amino acids, tryptophan, phenylalanine, and aspartic acid, are minor constituents of most tissues, yet are significant stimulants of food searching activity in the sea robin, *Prionotus carolinus* (Bardach and Case, 1965). Valine makes a substantial contribution to the feeding activity induced by amino acid mixtures in the carp, *Cyprinus carpio* (Saglio *et al.,* 1990). In the pinfish, *Lagodon rhomboides,* the minor amino acids aspartic acid, isoleucine, and phenylalanine are nonstimulatory alone, yet contribute markedly to the stimulatory capacity of a synthetic mixture (Carr and Chaney, 1976). Likewise, the nucleotide uridine 5′-monophosphate, a minor constituent of a worm extract, markedly increases the ingestion, by a juvenile eel (*Anguilla japonica*), of a diet containing a mixture of amino acids (Takeda *et al.,* 1984). An awareness that minor tissue constituents can make major contributions to the stimulatory capacity of natural mixtures is important in the analysis and preparation of synthetic stimulants for use in fish feeds or baits.

Feeding stimulants for herbivorous and carnivorous fish may be very different. In the herbivore *Tilapia zillii,* an organic acid, citric acid, plus the amino acids, glutamic acid, aspartic acid, serine, and lysine are major stimulants of feeding behavior (Johnsen and Adams, 1986; Adams *et al.,* 1988). Although the above substances are minor constituents of animal tissue, they are major components of romaine lettuce, a plant readily eaten by *Tilapia.* Likewise, dimethyl-β-propiothetin, a common constituent of green algae, is not reported in animal tissues, and serves as a potent feeding stimulant for herbivorous goldfish, carp, and *Tilapia* (*e.g.,* Nakajima *et al.,* 1989).

High concentrations of the low-molecular-weight substances shown in Table II are characteristic features of molluscs and crustaceans, and to a lesser extent, fishes, that are subjected to the stresses imposed by living at high or fluctuating salinities (Yancey *et al.,* 1982). These small organic compounds, together with inorganic ions, represent the major osmotically active solutes present in the cells of many marine animals. Six substances—glycine, alanine, proline, taurine, betaine, and trimethylamine oxide—are all among the most prominent organic solutes in the marine molluscs, crustaceans, and fishes analyzed in this study (see Figs. 2, 3). The above six substances are the dominant six components in the molluscan extracts; and five of them (exclusive of alanine) predominate in crustacean extracts. Likewise, four of these substances—TMO, taurine, alanine, and glycine—are among the top eight components in fish. Significantly, each of the substances listed above is a "compensatory solute," a term proposed to indicate the role of such molecules in maintaining the functional properties of enzymes and structural proteins against the destabilizing effects of common inorganic ions and some organic solutes (Bowlus and Somero, 1979; Yancey *et al.,* 1982; Clark, 1985; Yancey and Burg, 1990). Therefore, this description of the low molecular weight components in the tissues of 30 species of marine organisms also reveals the ubiquity of compensatory solutes and, interestingly, shows that each of these solutes is also a stimulant of feeding behavior in marine fish.

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Transdifferentiation of Larval Flagellated Cells to Choanocytes in the Metamorphosis of the Demosponge *Haliclona permollis*

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Abstract. The free-swimming parenchymella larvae of *Haliclona permollis* have a surface of flagellated cells that function in locomotion. These flagellated cells disappear from the larval surface soon after larval settlement, but the debate about their fate during metamorphosis has not been resolved. An improved method for electron microscopy enabled us to visualize minute ellipsoid granules characteristic of the larval flagellated cells of this sponge and to use these granules as natural markers to follow the fate of flagellated cells in metamorphs. On metamorphosis, the axonemes of the flagellated cells are withdrawn into the cell body and persist for some time, thus serving as a second natural marker for flagellated cells in postlarval forms.

Within 12 h after settlement, the metamorphs have both markers in amoeboid cells derived from flagellated cells. The minute ellipsoid granules are found in the amoeboid cells of the metamorphs 24 h after settlement, and in the choanocytes of the juveniles 36 h after settlement. Therefore, it is proposed that the choanocytes derive from the larval flagellated cells by way of an amoeboid cell stage. These results suggest that the flagellated cells of parenchymella larvae participate in the formation of juveniles.

Introduction

Most sponges, which are sessile as adults, have free-swimming larvae in their life cycles. The larvae use a surface layer of flagellated cells to swim around for a while, then settle on a substratum. Soon after the onset of sessile life, the locomotive organ disappears from the larval surface. Such metamorphs without flagella are chaotic in organization; they consist mainly of several types of amoeboid cells (Brien and Meewis, 1938). Sometime later, a canal system and choanocyte chambers are formed in the metamorphs. The fate of larval cells during metamorphosis and the origin of the choanocytes of juveniles have been debated for many years among sponge biologists (Fell, 1974; Simpson, 1984; Kaye and Reiswig, 1991; Woollacott, 1993).

In calcareous sponges, larval flagellated cells are transformed into the choanocytes of a juvenile during metamorphosis (Duboscq and Tuzet, 1937; Amano and Hori, 1992, 1993). In demosponges, however, two views have been presented: one is that the flagellated cells are transformed into choanocytes, as in calcareous sponges (Levi, 1956; Borojevic and Levi, 1965; Boury-Esnault, 1976); the other is that during metamorphosis the flagellated cells are lost by exfoliation or phagocytosis and not involved in the formation of juveniles (Brien and Meewis, 1938; Harrison and Cowden, 1975; Bergquist and Glasgow, 1986; Misevic *et al.*, 1990; Kaye and Reiswig, 1991). In the latter view, choanocytes are thought to derive from archeocytes.

Because larval flagellated cells change radically in morphology in the early stages of metamorphosis, it is almost impossible to follow their developmental fates unless the cells have some identification markers. We took advantage of two natural markers: the flagellar axoneme retracted in the cell body and the minute ellipsoid granules characteristic of the larval flagellated cells of *Haliclona permollis*. Because both of these markers are very small and difficult to visualize, a large number of good-quality elec-

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tron micrographs of metamorphosing larvae were necessary to reveal the transformation process of the larval flagellated cells. An improved fixation method (Amano and Hori, 1992, 1994) made it possible to obtain electron microscopic images of sponge metamorphs.

In this report, we show that larval flagellated cells transform into choanocytes during metamorphosis. This process of cellular transformation is thought to be another example of transdifferentiation (Schmid, 1992).

Materials and Methods

Sponges and larvae

In June 1993 and 1994, colonies of *H. permollis* were collected from rafts in Mutsu Bay in Aomori prefecture in northern Japan. They were immediately placed in containers with seawater, brought to the laboratory of Asamushi Marine Biological Station within 1 h, and kept in running seawater.

The exact taxonomy of our specimens is uncertain. Not only is *H. permollis* thought to be synonymous with *H. cinerea* (de Weerdt, 1986), but Japanese *H. permollis* is probably different from European *H. permollis* (or *H. cinerea*), and may be a new species. For the time being, the sponge used in this study should be referred to as *H. permollis sensu* Tanita. The sponge specimens of *H. permollis sensu* Tanita are now kept by us and are accessible on request. For brevity, however, we refer here to this Japanese sponge simply as *H. permollis.*

Haliclona permollis releases larvae in early summer in Japan. In the laboratory, only about one-fifth of the sponges released larvae, and most of these released larvae every day for 5 days or more. To collect larvae, we placed adults in still seawater at about 0530. Under natural illumination, larval release began soon after dawn and ceased at about 1100. Larvae began swimming as soon as they were released.

Electron microscopy

Free-swimming larvae could be picked up on a platinum loop and placed into fixative. Metamorphosing larvae were easily broken during fixation and dehydration and required different handling. Larvae that settled beneath the air-water interface were very adhesive and were easily attached to a small piece (about 1.0 × 2.0 mm) of membrane filter (Millipore, 0.45-μm pore size) that sank swiftly in media, making centrifugation unnecessary and reducing the mechanical stress on the larvae. After fixation and dehydration, the membrane filter was dissolved in propylene oxide, a clearing agent, so that the metamorphs could be embedded without the piece of filter.

Larvae and metamorphs attached to the small piece of filter were fixed in ice-cold 0.1 *M* cacodylate buffer (pH 7.4) containing 3.0% glutaraldehyde and 1.0% paraformaldehyde. For each 100 ml of the fixative, 14 g sucrose and 0.05 g anhydrous calcium chloride were added. After 2 h, samples were rinsed twice in 0.1 *M* cacodylate buffer and postfixed in 1.0% osmium tetroxide in 0.1 *M* cacodylate buffer (pH 7.4) for 1 h. Samples were dehydrated through a graded ethanol series, cleared in propylene oxide, and embedded in Spurr epoxy resin (Spurr, 1969). Semithin sections were stained with toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, then examined and photographed using a Hitachi H-500 electron microscope.

Results

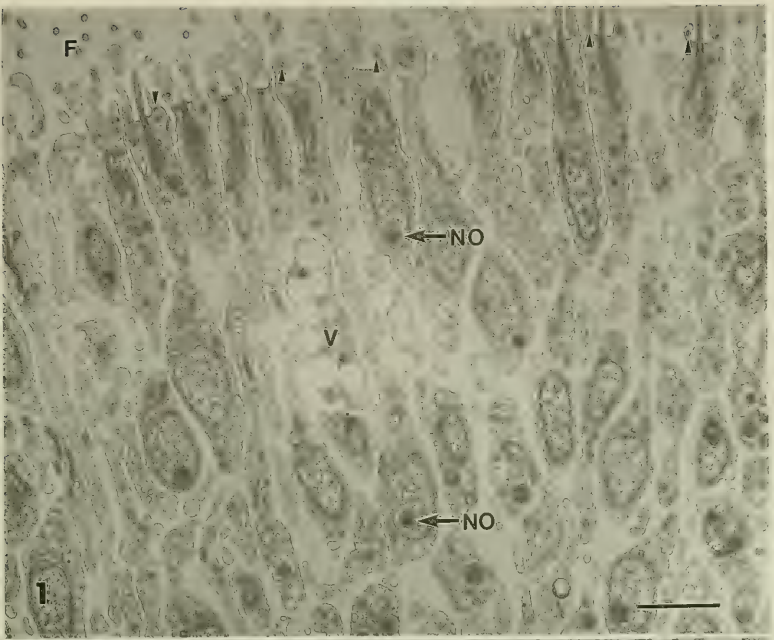
Flagellated cells of free-swimming larvae

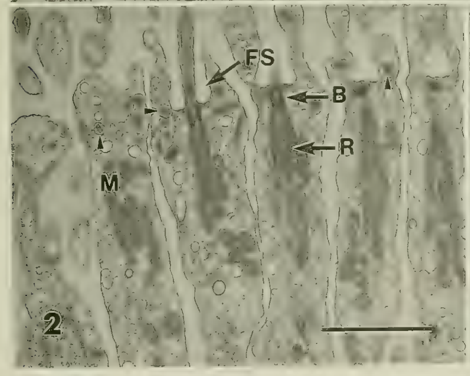
The free-swimming parenchymella larvae of *Haliclona permollis* are about 250 μm in length and 180 μm in width. Flagellated cells make up the entire larval surface, except at the posterior end where many reddish brown pigment granules occur in the cytoplasm.

Parenchymella larvae were fixed for electron microscopy on the morning of the day of their release. The larval surface region consists of a pseudostratified columnar epithelium of elongate flagellated cells; however, a basal lamina is absent (Fig. 1). At the free surface of these flagellated cells is a flagellar socket composed of a pit about 2.4 μm deep and 0.8 μm wide; a single flagellum emerges from the bottom of the socket. Flagella possess typical axonemes with the 9 + 2 arrangement of microtubules. The proximal end of the flagellar axoneme is somewhat electron-dense, terminating in a cylindrical basal body (Fig. 2). The long flagellar rootlet is seen as a tuft of filamentous matter without striations. Elongate mitochondria (about 1.2 × 0.3 μm) with well-developed cristae occur regularly around the rootlet.

Flagellated cells have the smallest nucleus (about 3.2 μm in length and 1.8 μm in width) among larval cell types. The nucleus is always located in the basal region of the cytoplasm, and the slender intermediate region of the cell connects the nuclear region with the apical region of the cell (Fig. 1). Thus the deeper the nucleus occurs, the longer the flagellated cell. We often found nucleolated flagellated cells, but the ratio of anucleolated to nucleolated is unknown. Flagellated cells contain a Golgi apparatus on the free-surface side of a nucleus, elliptical mitochondria, a few phagosomes, various vesicles, and free ribosomes.

We found minute electron-dense granules concentrated in the apical cytoplasm near the free surface of larval flagellated cells (Figs. 1, 2). These ellipsoid granules are always encapsulated by a limiting membrane and are usually smaller than 0.5 μm in length and 0.2 μm in width.





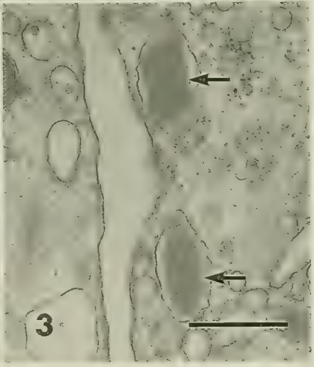


Figure 1. Pseudostratified columnar epithelium of elongate flagellated cells in the surface region of the free-swimming parenchymella larva of *Haliclona permollis*. Arrowheads: minute ellipsoid granules, NO: nucleoli, V: vesicular cell, F: flagellum. Scale bar = 3.0 μm.

Figure 2. Higher magnification of the flagellated cells in Figure 1. Electron-dense minute ellipsoid granules (arrowheads) occur in the apical cytoplasm of the cell. FS: flagellar socket, B: basal body, R: rootlet, M: elongate mitochondria. Scale bar = 2.0 μm.

Figure 3. Higher magnification of the minute ellipsoid granules (arrows) in the larval flagellated cells. These granules are composed of a crystalloid substance and covered by a limiting membrane. Scale bar = 0.5 μm.

Sometimes in section they appear square or cylindrical, but highly magnified images of these granules reveal a crystalloid substructure (Fig. 3). No minute ellipsoid granules are found in any larval cells except the flagellated cells.

Residual flagellar axoneme in amoeboid cells

After a free-swimming period of 12–36 h, larvae settled on the substratum (a glass surface or an air-water interface) and quickly transformed from an ellipsoid form to a convex disk. For a while after settlement, a gathering of reddish brown pigment granules originating from the posterior end of the larva was visible in the central region of the convex disk. These pigment granules disappeared within 12 h after settlement.

To observe the initial stages of metamorphosis, we fixed the settled larvae that still possessed concentrates of pigment granules (that is, were within 12 h after settlement) for electron microscopy. Figure 4 shows that flagellated cells are losing their characteristic morphology and the pseudostratified columnar epithelium is in the course of disorganization at the surface of such metamorphosing larva. Figure 5 shows a flagellar stub, a remnant of a flagellum on the cell body, at high magnification, and it is apparent that its flagellum was severed at the transitional region between the basal body and the proximal end of the axoneme. The excised flagellar axonemes were promptly retracted into the cell body (Figs. 4, 5). Only the axoneme without a flagellar membrane is withdrawn into the cytoplasm. As many as four sections of the axoneme were found per cell, so it must be coiled or cut into a number of pieces in the cytoplasm.

Amoeboid cells derived from flagellated cells migrate to the inner part of the metamorphosing larva; these amoeboid cells have residual flagellar axonemes (Figs. 6, 7). Some axonemes are in the process of rapid decomposition, but others still maintain the typical 9 + 2 microtubule construction (Fig. 7).

Figure 8 shows the distribution of diameter of the nuclei of nucleolated amoeboid cells and archeocytes in the metamorphs. As both of these cell types are amoeboid cells with pseudopodia and it is difficult to estimate their cell size precisely, the diameter of their nuclei was measured instead. Two peaks are evident in the size distribution of the nuclei, one at about 3.4 μm, and the other at 2.3 μm. By comparison with amoeboid cells in the larval interior, we conclude that the nucleolated cells whose nuclei are larger than 3.0 μm are archeocytes. These archeocytes are large cells with numerous phagosomes (Fig. 9), and residual axonemes never occur in such cells. In contrast, residual flagellar axonemes are observed in nucleolated cells whose nuclei are smaller than 3.0 μm (solid parts of the columns in Fig. 8). The proportion of cells with residual axonemes (7/41) among nucleolated amoeboid cells is thought to be an underestimate because the residual axoneme is small and easily overlooked. We conclude that two types of nucleolated cells occur in the metamorphosing larva: one is the archeocytes and the other is the amoeboid cells derived from the larval flagellated cells.

Minute ellipsoid granules in amoeboid cells and choanocytes

We have shown that minute ellipsoid granules are characteristic of the larval flagellated cells of *H. permollis* (Figs. 1, 2). In the initial stages of metamorphosis, those granules are found in amoeboid cells that have residual flagellar axonemes (Fig. 9). The nuclei of these cells are small (<3.0 μm) and have heterochromatin masses. The large archeocyte in Figure 9 has, however, neither minute ellipsoid granules nor residual axonemes. Figure 10 shows a nucleolated amoeboid cell with two sections of residual axoneme and a minute ellipsoid granule. The minute ellipsoid granule together with the residual axoneme evidently reveal the origin of this amoeboid cell.

Twenty-four hours after settlement, the metamorphosing larvae have a convex disk shape and appear chaotic in organization. The concentration of reddish brown pigment granules is no longer evident and spongin fiber formation has begun. Flagellar axonemes have already decomposed, but the minute ellipsoid granules characteristic of larval flagellated cells still occur in the larval cells at this stage of metamorphosis. In Figure 11, a nucleolated cell from a 24-h metamorph can be seen to contain the minute ellipsoid granules. This cell has a large nucleolus and phagosomes. Figure 12 shows similar amoeboid cells with minute ellipsoid granules, but no nucleus is visible in these cells. Phagosomes are more numerous in amoeboid cells with minute ellipsoid granules from 24-h metamorphs than from 12-h metamorphs.

About 36 h after settlement, flagellar chamber formation began in the metamorphosing larvae. Figure 13 shows a flagellar chamber in the course of development and one choanocyte with a minute ellipsoid granule. Figure 14 shows a granule in such a choanocyte at high magnification. We found fewer ellipsoid granules in 36-h metamorphs than in 24-h metamorphs. Figure 15 shows a more developed flagellar chamber in a metamorph 48 h after settlement. The flagellum of a choanocyte is encircled by a collar of microvilli which are swollen in places. The flagellar basal bodies are not associated with rootlets. The choanocytes usually contain a moderate number of phagosomes. Minute ellipsoid granules are not found in such choanocytes.

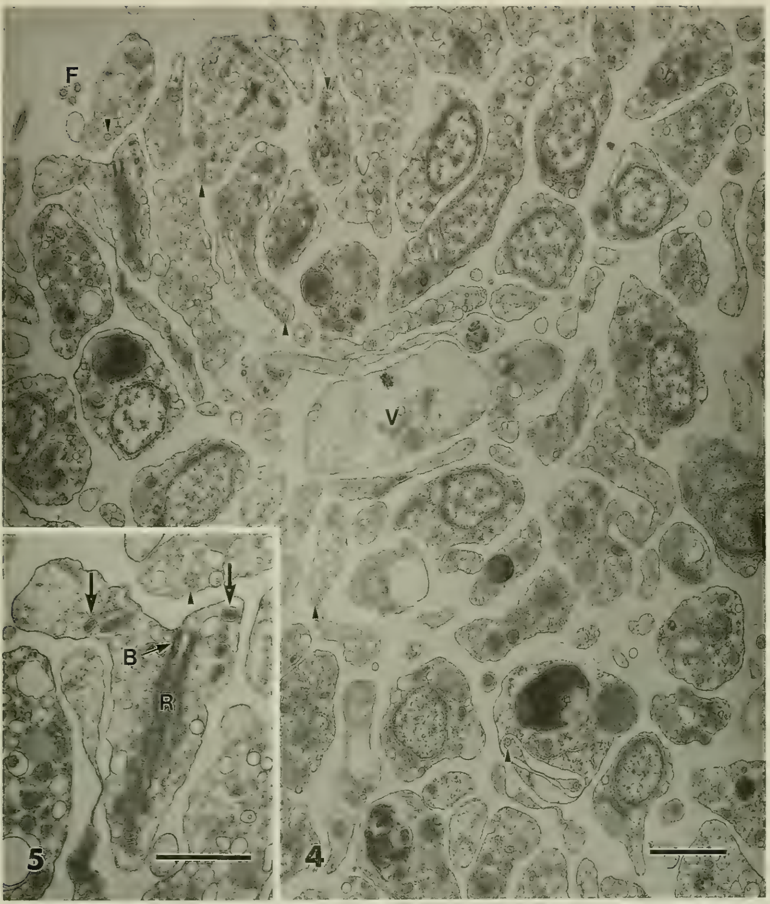


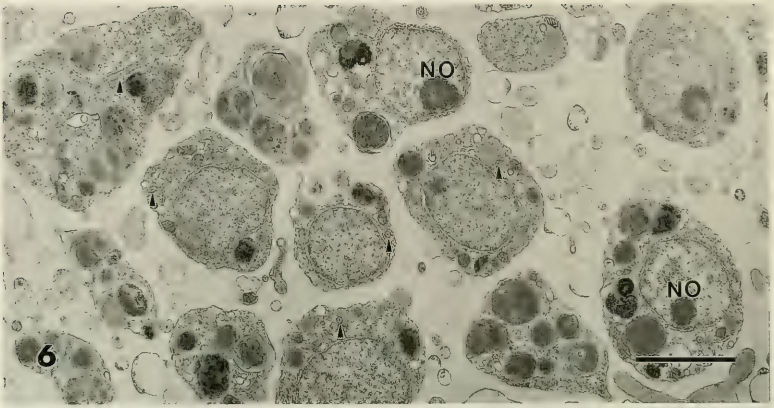
Figure 4. Surface region of the metamorphosing larva of *Haliclona permollis* soon after settlement. The pseudostratified epithelium of larval flagellated cells is in the course of disorganization. Flagellar axonemes (arrowheads) are seen in these cells. F: three sections of a coiled flagellar axoneme in a single membrane, V: vesicular cell. Scale bar = 2.0 μm.

Figure 5. Higher magnification of a transforming cell in Figure 4. This cell has lost its flagellum but retains a flagellar stub of a basal body (B) and a rootlet (R) near their original positions. Arrowhead: axonemal complex, arrows: minute ellipsoid granules. Scale bar = 1.0 μm.

Discussion

Two natural markers

Because the flagellated cells of sponge larvae change their anatomy very quickly and markedly during metamorphosis, it is difficult to elucidate the steps of this transformation. In this study, therefore, we employed two natural markers. One of these, the flagellar axoneme retracted in the cell body, was rapidly decomposed and all of them disappeared from the cell between 12 and 24 h after larval settlement. The other marker, the presence of minute ellipsoid granules, is



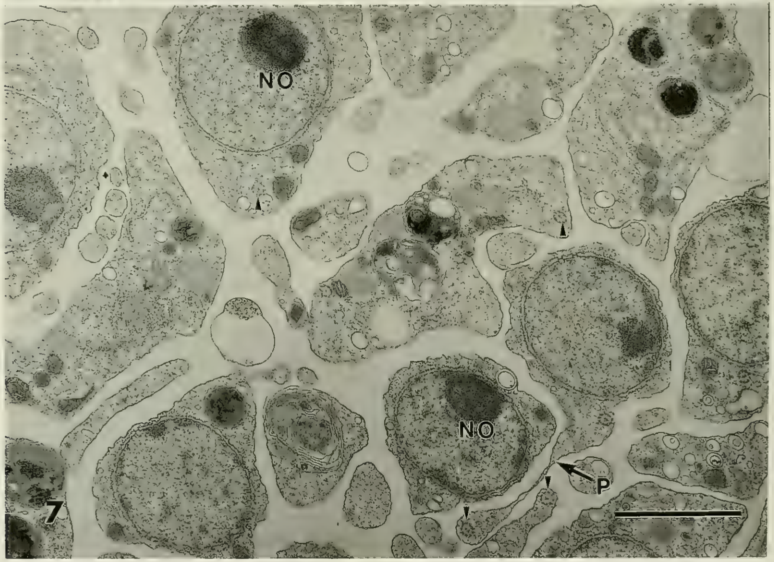


Figure 6. Amoeboid cells with residual flagellar axonemes (arrowheads) in the metamorphosing larva of *Haliclona permollis* within 12 h after settlement. NO: nucleolus. Scale bar = 2.0 μm.

Figure 7. Residual flagellar axonemes (arrowheads) occur in amoeboid cells within 12 h after settlement. The typical 9 + 2 microtubule construction of the axonemal complex is visible. NO: nucleolus, P: pseudopodium. Scale bar = 2.0 μm.

characteristic of the larval flagellated cells of *Haliclona permollis,* but similar granules were not found in the larvae of another *Haliclona* sp. (Amano and Hori, 1994). The minute ellipsoid granules are composed of a crystalloid substance, but their chemical nature is unknown. These granules disappeared from flagellated cells between 36 and 48 h after settlement. Therefore, in future studies, the fate of the larval flagellated cells should be traced for longer durations, and also in other sponges.

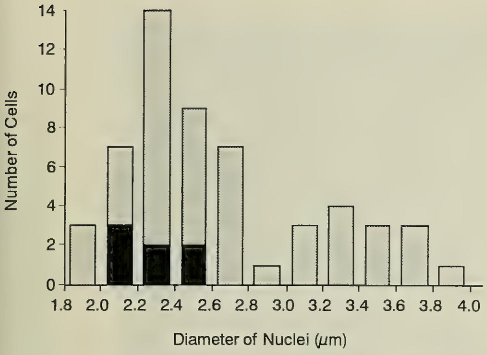


Figure 8. Distribution of the diameter of the nuclei of nucleolated amoeboid cells and archeocytes in the *Haliclona permollis* larvae in the initial stages of metamorphosis. The solid parts of the columns indicate the cells with residual flagellar axonemes, which are found in some of the nucleolated amoeboid cells but not in the archeocytes. The diameter of nuclei = (the major axis + the minor axis) ÷ 2. $n = 55$.

Loss of flagella

Soon after settlement, sponge larvae lose the flagella in the surface layer of cells. In *H. permollis,* the flagella are severed at the transitional region between the basal body and the proximal end of the axoneme, as in other animals (Blum, 1971; Sanders and Salisbury, 1989). In the amphiblastula larvae of calcareous sponges, flagellated cells shed and discard their excised flagella (Amano and Hori, 1993). In the parenchymella larvae of *H. permollis,* however, the flagellated cells withdrew the excised flagellar axonemes into the cell body. This latter course may be common among demosponges because similar internalized axonemes were observed in the metamorphosing larvae of other demosponges (Levi, 1964; Boury-Esnault, 1976). Thus, the residual flagellar axonemes are found in the amoeboid cells of metamorphosing parenchymellae, but not in those of the metamorphosing amphiblastulae.

Amoeboid cells with minute ellipsoid granules and axonemes in the cell body

De-flagellated larval epithelia do not invaginate in a cell sheet, but are disarranged into individual amoeboid cells that each migrate to the inner region of a metamorph by amoeboid movement. Two natural markers are found in the amoeboid cells of metamorphs within 12 h after settlement. These cells retain a basal body and a rootlet in the initial stage of metamorphosis, but these intracellular components of flagella disappear rapidly from the cytoplasm. The minute ellipsoid granules that were concentrated in the apical region of larval flagellated cells are dispersed in the cytoplasm of the amoeboid cells. It is obvious that the amoeboid cells with these markers derive from larval flagellated cells. Like archeocytes, some of the amoeboid cells have a nucleolus, but these cells are smaller and have fewer phagosomes than the archeocytes. It is apparent that the archeocytes have no residual axonemes or minute ellipsoid granules.

The flagellar axoneme observed in the amoeboid cells is evidently the one that was retracted in the cell body of the flagellated cells. The alternative possibility—that it may represent a flagellated cell phagocytosed by other larval cells or archeocytes—is excluded for the following reasons. (1) The flagellar axoneme, excised at the transitional region, is found in cells with a residual basal body and a rootlet. (2) In the cytoplasm of the amoeboid cells, the flagellar axoneme invariably occurs naked, without a covering membrane, and is never found in phagosomes. This is because only the axoneme, not the flagellar membrane, is severed and taken into the cell body. Axonemes without covering membranes are not found in the intercellular space of metamorphosing larvae. (3) In the early stages of metamorphosis, the flagellar axonemes are found only in amoeboid cells whose nuclei are smaller than 3.0 μm. Archeocytes, whose nuclei are larger than 3.0 μm, are phagocytes with many large phagosomes, but no flagellar axonemes occur within these cells or especially within their phagosomes.

Origin of choanocytes in H. permollis

Although residual axonemes have already decomposed 24 h after settlement, minute ellipsoid granules still occur in amoeboid cells that have migrated into the inner region of metamorphs. Because these amoeboid cells usually have more phagosomes than those in earlier stages, they are probably growing in the metamorphs. We suggest that such amoeboid cells are precursor cells of choanocytes.

Minute ellipsoid granules were found in choanocytes that were still in the course of differentiation about 36 h after settlement. It is likely that these choanocytes derive from amoeboid cells originated from flagellated cells, but the possibility that some of the choanocytes originate from archeocytes cannot be excluded. Because large numbers of choanocytes are necessary for the flagellar chamber formation of juveniles, multiple progenitor cell types cannot be ruled out. About the time of flagellar chamber formation, the number of minute ellipsoid granules decreases rapidly, and these granules are not found in fully developed choanocytes 48 h after onset of metamorphosis. On the basis of our findings, we suggest that larval flagellated cells are transformed into the choanocytes of the juvenile of *H. permollis* by the process illustrated in Figure 16.

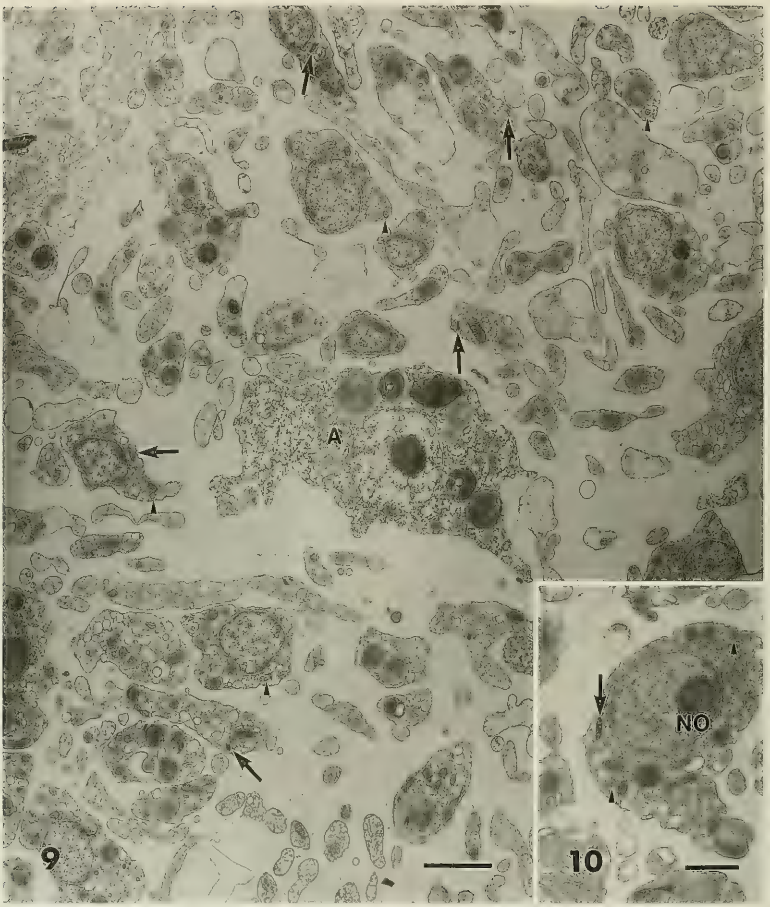
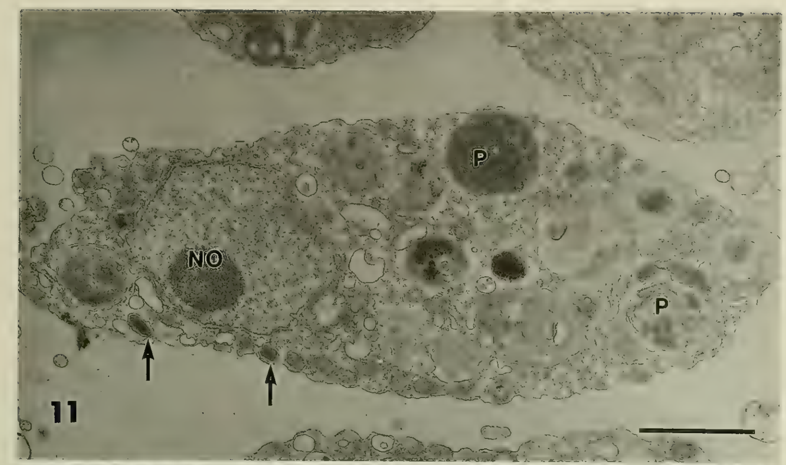


Figure 9. Small amoeboid cells and an archeocyte in the early stage of the metamorphosis of *Haliclona permollis.* Residual axonemes (arrowheads) and minute ellipsoid granules (arrows) occur in the small amoeboid cells but not in the archeocyte (A). Scale bar = 2.0 μm.

Figure 10. Nucleolated amoeboid cell with one minute ellipsoid granule (arrow) and two sections of axonemal complex (arrowheads). Both of these natural markers distinctly show the origin of this cell. NO: nucleolus. Scale bar = 1.0 μm.

Origin of choanocytes in other sponges

In calcareous sponges, the consensus is that the choanocytes of juveniles derive from larval flagellated cells (Duboscq and Tuzet, 1937; Amano and Hori, 1993). Similarly, in the demosponge *Polymastia robusta,* the choanocytes probably derive from larval flagellated cells, because the blastula larva of this sponge consists only of a layer of flagellated cells (Borojevic, 1967). Brien and Meewis (1938) studied the process of metamorphosis in *Ephydatia fluviatilis,* a freshwater sponge, and concluded that all larval flagellated cells are phagocytosed by archeocytes and play no role in the formation of juvenile sponges. It is likely, however, that light microscopy lacked sufficient resolution to show whether the amoeboid cells derived



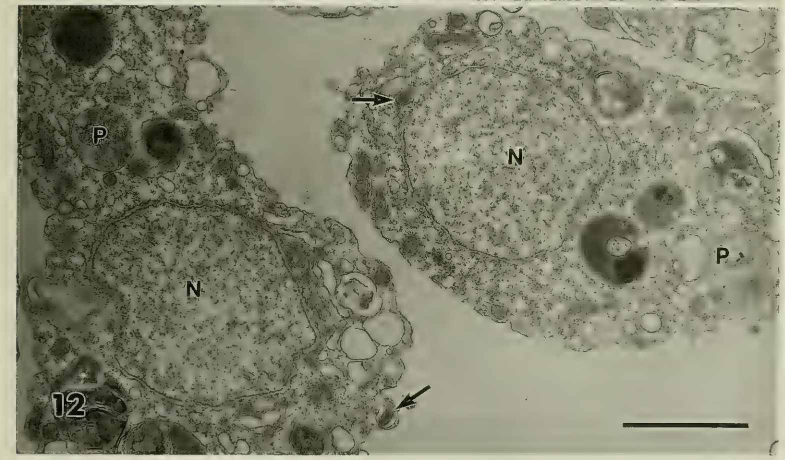
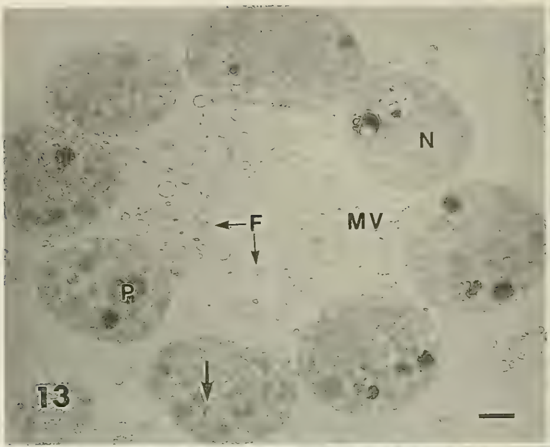


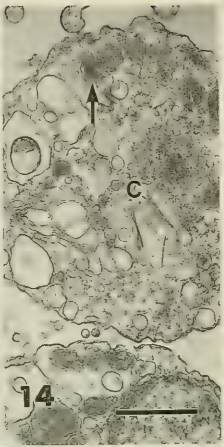
Figure 11. An amoeboid cell with two minute ellipsoid granules (arrows) in the metamorph of *Haliclona permollis* about 24 h after settlement. This cell has a nucleolus (NO) and phagosomes (P). Scale bar = 2.0 μm.

Figure 12. Amoeboid cells with minute ellipsoid granules (arrows) about 24 h after settlement. Nucleoli are not visible in these cells. N: nuclei, P: phagosome. Scale bar = 2.0 μm.

from the flagellated cells were positioned within or close to the archeocytes. In *Microciona prolifera,* Misevic *et al.* (1990) labeled the flagellated cells of free-swimming larvae with ^{125}I. The label was found in the phagosomes of archeocytes in the metamorphs, but not in the choanocytes of juveniles, leading the authors to conclude that all of the flagellated cells were phagocytosed by archeocytes during metamorphosis and did not give rise to choanocytes. The origin of the choanocytes was, however, not shown because the label was not found in the phagosomes of choanocytes, which are thought to derive from archeocytes.

Our conclusions in the present paper are not contrary to the concept that the archeocytes are multipotential and capable of differentiating into choanocytes. In cases where a supply of larval flagellated cells is not available, choano-





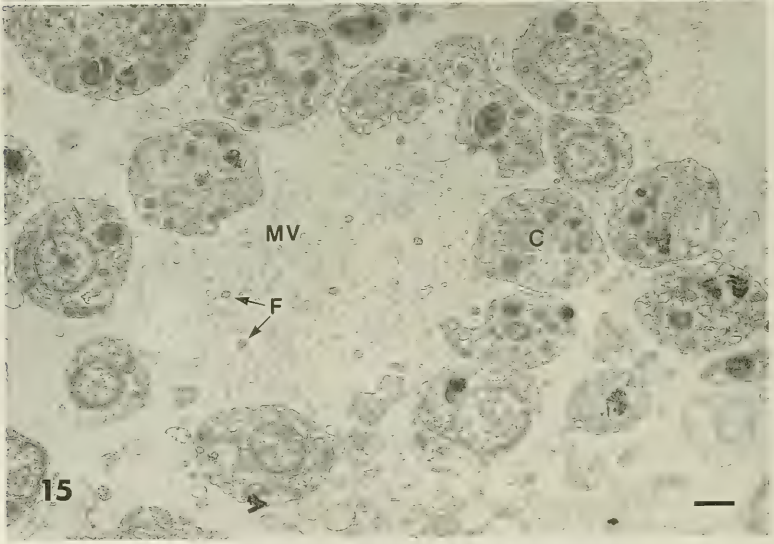


Figure 13. Early stage of flagellar chamber formation in the juvenile of *Haliclona permollis* about 36 h after larval settlement. The minute ellipsoid granule (arrow) occurs in the choanocytes with a collar of microvilli (MV) and a flagellum (F). N: nucleus, P: phagosome. Scale bar = 2.0 μm.

Figure 14. Minute ellipsoid granule (arrow) in a choanocyte (C) at high magnification. Scale bar = 1.0 μm.

Figure 15. A flagellar chamber of the juvenile of *Haliclona permollis* 48 h after larval settlement. The flagella (F) of choanocytes (C) are associated with a basal body, but without a rootlet. Microvilli (MV) of the collar are swollen in places. Scale bar = 2.0 μm.

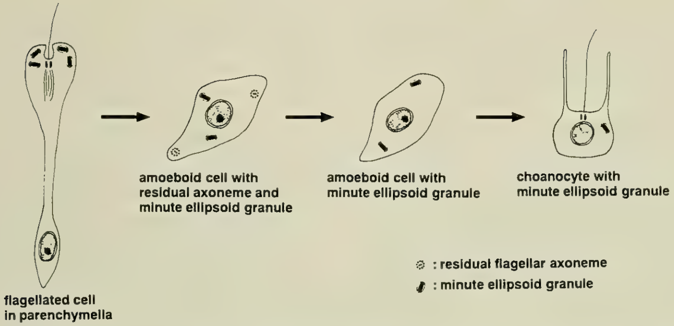


Figure 16. Transformation of larval flagellated cells into the choanocytes of juveniles in the metamorphosis of *Haliclona permollis.* The stages of the transformation (represented by arrows) are based on the results obtained by observing two natural markers, the residual flagellar axonemes and the minute ellipsoid granules. Soon after the settlement of the larvae, the flagellated cells dedifferentiate to the amoeboid cells that have both markers. The axonemes diappear between 12 and 24 h after settlement. In juveniles, the amoeboid cells differentiate to choanocytes that retain the ellipsoid granule. This granule vanishes between 36 and 48 h after the onset of metamorphosis, so it is not found in the choanocytes of the adult sponge.

cytes can derive from archeocytes. For example, within the free-swimming larvae of *Ephydatia fluviatilis,* choanocytes should be differentiated from the archeocytes precociously before larval settlement (Brien and Meewis, 1938). Also, in juvenile sponges reconstituted from the excised central part of parenchymella larvae (Borojevic, 1966) or from cell aggregates of purified archeocytes (Buscema *et al.,* 1980), choanocytes evidently derive from archeocytes. In contrast, when sufficient numbers of choanocytes are supplied by larval flagellated cells, the archeocyte differentiation to choanocytes is probably unnecessary. In *H. permollis,* some surplus larval flagellated cells seem to be degenerated and phagocytosed by other larval cells during metamorphosis, because we found only a few picnotic cells in metamorphs. Borojevic (1966) argued that the archeocytes are reserved embryonal cells that regulate the equilibrium between the different categories of cells. The archeocytes should eliminate excessive cells by phagocytosis and, on the other hand, differentiate into deficient cell types to fill the shortage.

Transdifferentiation

Transdifferentiation is the process by which cells that have already expressed specific differentiated traits change into another cell type distinguished from the original cells by a set of phenotypic characters (Okada, 1991; Schmid, 1992). It has been demonstrated experimentally that fully specialized somatic cells can undergo a change in their cellular commitment and gene expression. Under certain culture conditions, the retinal pigment epithelium of chick embryos can transdifferentiate to lentoid cells that express typical lens crystallins (Itoh and Eguchi, 1986). In a medusa, Schmid and Alder (1984) showed that striated muscle cells can undergo pluripotent transdifferentiation and form a complex regenerate under some experimental conditions.

In the early stages of the metamorphosis of *H. permollis,* the highly differentiated flagellated cells of larvae dedifferentiate to amoeboid cells that subsequently differentiate to choanocytes in juveniles. The process of this transformation is not simply the regeneration of an excised flagellum (Sanders and Salisbury, 1989), because the flagellar basal body and rootlet have been completely lost in the amoeboid cells. Thus the results of the present study demonstrate that the flagellated cells of *H. permollis* larvae transdifferentiate into the choanocytes of juveniles by way of an intermediate amoeboid cell stage.

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Endogenous β-Galactosidase Activity in the Larval, Pupal, and Adult Stages of the Fruit Fly, *Drosophila melanogaster,* Indicates Need for Caution in *lacZ* Fusion-Gene Studies

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Abstract. β-galactosidase activity is known to exist in *Drosophila melanogaster,* but a detailed analysis of the tissue-specific patterns of activity has not previously been reported. Such an analysis is of particular interest because *Drosophila* is commonly used for making transformants that carry fusion genes in which the *E. coli* β-galactosidase gene, *lacZ,* is used as a reporter gene. When these transformants are analyzed for β-galactosidase activity by using chromogen X-gal staining, the method does not distinguish true fusion-gene activity from endogenous β-galactosidase activity or from the β-galactosidase activity of bacterial contaminants. Therefore, detailed maps of endogenous β-galactosidase activity in this organism would help to prevent errors in data interpretation and would indicate which stages were most appropriate for experiments with the *lacZ* transformants. We have constructed such maps by applying X-gal staining methods to serial frozen sections and whole mounts of larval, prepupal, pupal, and adult stages of *D. melanogaster* reared under axenic conditions. Results showed endogenous β-galactosidase activity in a variety of organs including the larval intestine, spiracles, lymph glands, cellular epidermis, and eye-antenna imaginal discs; the pupal cellular epidermis, lymph glands, imaginal tissues, fat body, and spiracle; and the adult pericardial cells, thoracic nephrocytes, ventriculus, and reproductive system. The good correlation between staining and metamorphic remodeling and phagocytic activity indicates that endogenous β-galactosidase is physiologically interesting.

Introduction

The glycosidase β-galactosidase is known to occur in a variety of microorganisms, plants, and animals (Wallenfels and Weil, 1972), and the fruit fly *Drosophila melanogaster* is no exception (Knipple and MacIntyre, 1984; Fuerst *et al.,* 1987; Glaser *et al.,* 1986). This enzyme, which cleaves the β-glycosidic bond between D-galactose and other sugar moieties, has been extensively studied in the prokaryote *Escherichia coli,* where it cleaves lactose into glucose and galactose.

With the advent of recombinant DNA technology, *E. coli* β-galactosidase and its structural gene, *lacZ,* have become very useful in molecular studies. Because *lacZ* has been sequenced and cloned, and produces an enzyme easily detectable with various chromogenic assays, it is used extensively as a reporter gene to trace the spatial and temporal expression of genes in transgenic organisms. Before it is inserted into another genome, the *E. coli lacZ* reporter gene is fused to the promoter, with or without additional sequences, of a gene whose expression is to be monitored. When this *lacZ* fusion gene becomes incorporated into the genome of an organism, its expression can be detected by assaying for *E. coli* β-galactosidase. Once the elegant P-element-mediated mutagenesis technique was developed for inserting genes into the genome of *Drosophila melanogaster* (for review see Spradling, 1986), the fruit fly became a perfect recipient for *lacZ* fusion genes, and transformed flies with fusion genes containing an *E. coli lacZ* reporter gene became common.

Typically in these transformed flies, tissues are assayed for the reporter gene product, *E. coli* β-galactosidase, by using the chromogen X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Here, β-galactosidase hydrolyzes the β-glycosidic bond of X-gal and produces an insoluble blue stain of indolyl monomers. Because minute amounts of enzyme can be detected with the X-gal stain, it is an effective method for tagging fusion-gene expression. The

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lacZ fusion gene/X-gal system is an economical and rapid assay of tissue-specific gene activity in *D. melanogaster*; it is even more sensitive than immunocytochemical (Ghysen and O'Kane, 1989) or *in situ* hybridization techniques.

However, when assaying *E. coli lacZ* fusion-gene activity with X-gal, it is important to remember that these transformed fruit flies will express an endogenous β-galactosidase as well as the *E. coli* enzyme. Where the endogenous β-galactosidase is present, it will hydrolyze X-gal and will be indistinguishable from its ectopic counterpart—even when the pH of the staining buffer has been adjusted to levels appropriate for optimal *E. coli* β-galactosidase activity (Ashburner, 1989).

An endogenous β-galactosidase, β-galactosidase-1, has been isolated from adult *D. melanogaster* (Knipple and MacIntyre, 1984) and characterized as a glycoprotein homodimer with an optimal activity (hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside) at a pH of 6.0 (Fuerst *et al.,* 1987). Furthermore, three electrophoretically distinct minor forms of the enzyme have been found in other developmental stages of *D. melanogaster* (Knipple, 1983). Despite these findings, endogenous β-galactosidase expression has been thought to be negligible and has not been regarded as a problem in *lacZ* fusion-gene studies in *D. melanogaster* (see, for example, Lawrence, 1992, p. 52).

In this study, we show that endogenous β-galactosidase activity is not negligible. Using an X-gal assay identical to those employed to detect *E. coli lacZ* fusion-gene products, we identified the tissues that express endogenous β-galactosidase activity in the late third-instar larva, prepupa, pupa, and adult of wild-type *D. melanogaster*. Specifically, we assayed for β-galactosidase activity in active and nonactive third instar larvae: 15 metamorphic stages; and newly eclosed adults and those at age intervals of 1 to 2, 3 to 5, and 7 to 10 days. The resulting maps of expression not only provide a guide to endogenous activity for investigators using a *lacZ* reporter gene in a wild-type background, but also characterize the distribution and intensity of endogenous β-galactosidase activity in different life stages, and suggest that endogenous β-galactosidase is intrinsically interesting in view of its association with metamorphic remodeling and phagocytic activity.

Materials and Methods

Axenic cultures

To prevent background staining due to commensals or other microorganisms that might express β-galactosidase, we reared a wild-type Gailey strain of *D. melanogaster* (a Canton-S derivative, kindly supplied by Dr. Donald Gailey of California State University at Hayward) under sterile conditions (modified from Roberts, 1986). Mature adult flies were placed on fresh cornmeal/molasses/agar/yeast/malt medium (with proprionic acid added as a mold inhibitor) for 3–6 hours. Eggs were collected, placed in vials of fine-mesh plankton net, and sterilized in a solution of 5% sodium hypochorite (full-strength Clorox). After the chorion had sloughed off (10–15 min) and hatched larvae (if any) were removed, the eggs were rinsed three times in sterile distilled water, 10 min each rinse. A sterile pasteur pipette was used to transfer eggs to autoclaved vials of axenic medium (1.5 g agar/7 g cornmeal/7 g yeast/1 ml proprionic acid to 100 ml water). All egg-handling operations were carried out in a sterile laminar flow hood, and culture vials were stored in the sterile hood until sampling time.

Collection of developmental stages

Late third-instar larvae were collected as they crawled out of the food medium and were sampled as two groups: actively crawling larvae and older nonactive larvae just prior to eversion of the spiracles. We chose these particular groups of late third-instar larvae because they are often collected for sampling of larval tissues and imaginal discs. The only visible difference between the two groups is crawling activity; however, changes in endogenous β-galactosidase activity may take place in the nonactive larvae as they approach the prepupal stage. To track the tissue-specific activity of β-galactosidase through metamorphosis, we collected both prepupal and pupal stages (P1–P15, Bainbridge and Bownes, 1981). To determine whether the enzyme activity in adults varied with age or sex, we collected male and female flies at intervals of 0.5–3 h, 1–2 days, 3–5 days, and 7–10 days after eclosion.

Whole mounts

Whole-mount preparations were dissected from larval, pupal, and adult stages and stained with X-gal. To determine whether staining varied among individual animals, we dissected and stained 10 animals from each of the following stages: active and inactive late third-instar larvae: prepupal stages P1–P3: and newly eclosed adults and adults aged 1 to 2 days, 3 to 5 days, and 7 to 10 days. Older pupal stages were too fragile to dissect; thus they were not stained as whole mounts.

Animals were placed under a Nikon SMZ-U dissecting microscope and dissected with the aid of microforceps, iridectomy scissors, and 0.1-mm insect pins. Tools, pins, and small plastic petri dishes with a Silgard (Dow Corning Corp.) base were disinfected in 95% ethanol, and tissues were kept wet with phosphate-buffered saline (PBS), pH 7.2, that had been sterilized through a Gelman acrodisc filter assembly (0.2-μm poresize). Adult flies were anesthetized on ice or with ether before dissection. Any fat-body tissue surrounding organs was removed because it hardened during fixation, making identification of structures difficult.

Cryostat sectioning

Frozen serial sections (transverse sections of larvae and sagittal sections of pupae from stages P1–P15) were cut and stained with X-gal. Before they were frozen, pupae were removed from the puparia: super glue gel "Quick Gel" (Loctite Corp.) was used to adhere the ventral surface of the puparium to a petri dish; after the glue hardened, microforceps were used to dissect the puparium away from the pupa. This technique allowed rapid removal and accurate staging of the delicate pupa without damage. With the puparium removed, the pupal cuticle freezes in direct contact with the mounting medium and is firmly embedded within the block, allowing high-quality cryostat sections to be cut. For sectioning, the organisms were mounted in OTC (Miles Inc.) or TBS (Triangle Biomedical Sciences) on stubs cooled with dry ice. When storage before sectioning was necessary, mounted specimens were removed from the stubs and stored in airtight plastic bags at −80°C. We found that the resolution of X-gal staining was optimal in thick (20-μm) sections. Serial sections were cut on a Reichert-Jung 975C cryostat microtome at −18° to −21°C, collected in order on gelatin-coated slides, and air-dried for 1–3 h before fixation and X-gal staining.

Fixation

Whole mounts and cryostat sections were fixed according to published protocols (Liu *et al.*, 1988, and Glaser *et al.*, 1986) specifically designed for X-gal staining of *D. melanogaster* tissues.

Animals were immersed in freshly prepared 1.0% glutaraldehyde in PBS during dissection and were left in fixative at room temperature for an additional 15 min before a rinse in PBS (modified from Glaser *et al.*, 1986). We found it necessary to dissect the animals thoroughly for adequate fixative penetration. Tissues were fixed and rinsed directly on the Silgard plates.

After air-drying, cryostat sections were fixed in freshly prepared 0.5% glutaraldehyde in PBS for 10 min at room temperature and were rinsed in PBS three times, for 10 min each time (after Liu *et al.*, 1988).

Staining procedure

Whole mounts and cryostat sections were stained with X-gal (protocol after Liu *et al.*, 1988) in a staining buffer; duplicate sets were processed at pH 7.2—appropriate for the optimal activity of *E. coli* β-galactosidase—and at pH 6.0—appropriate for the endogenous β-gal-1 enzyme (Fuerst *et al.*, 1987). Any visible staining of the endogenous β-galactosidase activity at pH 7.2 can be directly applied as expected background activity in X-gal staining assays designed to detect *lacZ* fusion gene activity.

In developing our methods, it became apparent that the X-gal should only be dissolved in *N,N*-dimethyl formamide immediately before staining. Once dissolved in the solvent, the X-gal begins to deteriorate (even when stored at −20°C) and staining intensity is reduced. If the age of the X-gal/*N,N*-dimethyl formamide solution is not constant between different samples, any quantitative measurement (albeit rough) of enzyme activity is compromised.

For comparison of enzyme activity in cryostat sections at the two pH levels, serial sections were collected alternately between two slides. One slide was then stained at pH 7.2 and the other at pH 6.0. This allowed precise comparisons between regions.

After fixation, cryostat sections were encircled with a hydrophobic barrier (Pap Pen, Kiota International Industries), immediately covered with a droplet of freshly prepared X-gal staining solution, and incubated in humidified covered trays for 16 h at 37°C. The X-gal solution was removed, and the sections were rinsed three times, 10 min each rinse, in PBS and mounted in Gel-Mount (Biomedia Corp.). Slides were stored in the dark at 4°C to preserve staining intensity (Glaser *et al.*, 1986).

After being stained and photographed, sections of the pupal stages were restained in hematoxylin/eosin. These additional stains were helpful in identifying various developing organs during metamorphosis. To prepare the slides for restaining, coverslips were removed by soaking the slides in PBS for up to 4 days at 4°C. After a 2-min rinse in water, sections were stained in Harris' hematoxylin (Luna, 1968) for 2 min, rinsed in tap water for 2 min, and rinsed through a graded series of ethanol (70%–90%). Sections were stained in eosin (a saturated solution of eosin in 95% ethanol) for 5 min. Dehydration was continued through 100% ethanol; sections were then cleared in toluene and mounted in Histoclad (Becton, Dickinson and Co.).

Animals dissected and fixed for whole-mount staining were given a final 15-min rinse in PBS and a brief rinse in staining buffer. Dissections were left pinned out on the Silgard plates and were immersed in X-gal staining solution, covered with a petri lid, placed in humidified covered trays, and incubated for 16 h at 37°C.

After staining, dissections were rinsed in PBS three times, 15 min each rinse. Organs of interest were removed and photographed as whole mounts. In the dissections stored in PBS in the dark at 4°C for a few days, the staining intensity remained the same, but afterwards some stain began to diffuse from intensely stained regions into adjacent nonstained tissues. For this reason, dissections were viewed within 2 days of staining. For long-term storage, whole mounts were stored in 100% glycerol at 4°C (Glaser *et al.*, 1986).

Photomicrography

Sections and whole-mount preparations were examined using bright-field microscopy and photographed on a Leitz

Dialux 20 microscope fitted with a Wild Photoautomat MPS 55 loaded with Kodak or Fuji 200 ASA color print film.

Transmission electron microscopy

To determine whether microorganisms were indeed eliminated from the gut in axenically reared animals, we first placed intestinal smears on nutrient and EMB agar media (Difco Laboratories). Microorganisms did not grow on these culture plates. We then viewed sections of the larval midintestine that exhibited β-galactosidase activity under the transmission electron microscope. This ultrastructural examination afforded an additional control: commensals present in the lumen and specialized to that environment might not grow on culture plates, but commensals and mycetocytes, insect cells that incorporate microorganisms (Chapman, 1969), can be distinguished at the ultrastructural level (Smith, 1968).

Stained segments of the larval midintestine were excised and stored temporarily in PBS with $CaCl_2$ at 4°C in plankton-net vials. Tissues were osmicated in a solution of 2% osmium tetroxide in PBS on ice for 1 h, rinsed in distilled water twice, and left in 70% ethanol overnight. Tissues were given two 7-min rinses, in 70% ethanol on ice; rinsed in 95% ethanol, 100% ethanol and propylene oxide three times, 7 min each rinse, at room temperature; and placed in a 1:1 solution of propylene oxide and Epon-Araldite epoxy resin in open vials for overnight infiltration.

Tissues were transferred to a drop of fresh resin and placed in an evacuated cool oven twice, for 20 min each time. Specimens were embedded in fresh resin and cured for 24 h at 60°C. Blocks were sectioned on a Sorvall Porter-Blum MT2-B ultramicrotome using glass knives broken on an LKB KnifeMaker. Thick sections cut in the transverse plane through regions of midintestine that stained with X-gal retained a light blue stain on the lumenal surface. To enhance contrast, thick sections were stained with toluidine blue and mounted in Permount (Fisher Scientific).

After screening the thick sections, we cut gold-to-silver ultrathin sections from the blocks. Sections were placed on grids, stained with a saturated solution of uranyl acetate in 100% ethanol for 20–30 min, rinsed with distilled water, stained in a 2% solution of lead citrate for 4 min, and rinsed in distilled water. After the grids were air-dried, sections were viewed on a Phillips CM 10 transmission electron microscope.

Results

Axenic cultures

Axenic conditions did not alter development in *D. melanogaster* except to slow the timing of larval development. This effect has been noted previously in axenic or mono-axenic cultures (Ashburner and Thompson, 1978). Compared to nonaxenic cultures, it took approximately 24 h longer for axenic cultures to reach the wandering third-instar stage. In all other respects, the axenic cultures appeared healthy and normal; the sterile conditions did not visibly affect the size or appearance of the larval, prepupal, pupal, or adult stages.

Transmission electron microscopy

Transverse sections through regions of the late third-instar larval midintestine were viewed by transmission electron microscopy. These sections of midintestine, including nonstained sections and those exhibiting intense X-gal staining associated with the lumenal surface, did not contain bacteria. The sections were inspected at several magnifications (from 6000 to 15,000×), and there was no evidence of bacterial flora residing in the lumen or housed intracellularly in mycetocytes (Fig. 1).

Staining of endogenous β-galactosidase activity at pH 7.2

Late third-instar larval stages. In the late third-instar larva, X-gal staining of β-galactosidase activity was found in the anterior and posterior spiracles, larval midintestine, lymph glands, cellular epidermis and in imaginal discs of the eye and antenna (Fig. 2). The only difference between the staining patterns of the active-roaming and the later inactive third-instar larvae was in staining intensity.

In active-roaming late third-instar larvae, intense staining was detected in the region of the anterior spiracles, specifically localized to the atria and region of the primordial dorsal prothoracic (or humoral) discs and spiracular glands. Two regions of intense staining were clearly demarcated in the midintestine: one just posterior to the proventriculus and the other just anterior to the junction between the midintestine and hindintestine. Cryostat-frozen and thick-plastic transverse sections through these midintestinal regions showed that the staining was associated with the lumenal surface of the intestinal epithelium. Light staining was found in the larval lymph glands (Fig. 3) and eye and antenna imaginal discs of some active larvae (Fig. 4). There was light staining of the inner cellular epidermis (Fig. 5).

In inactive late third-instar larvae, which are entering the quiescent stage just preceding pupariation, staining of the eye and antenna imaginal discs (Fig. 5A), lymph glands (Fig. 5B, C), and cellular epidermis (Fig. 5D, E) was noticeably more intense than in active third-instar larvae.

Prepupal stages (P1–P4). In white prepupae (P1), whole mounts showed a further increase in the staining intensity of the inner cellular epidermis, eye and antenna discs and lymph glands. Staining in the midintestine persisted. Staining in the now-everted anterior and posterior spi-

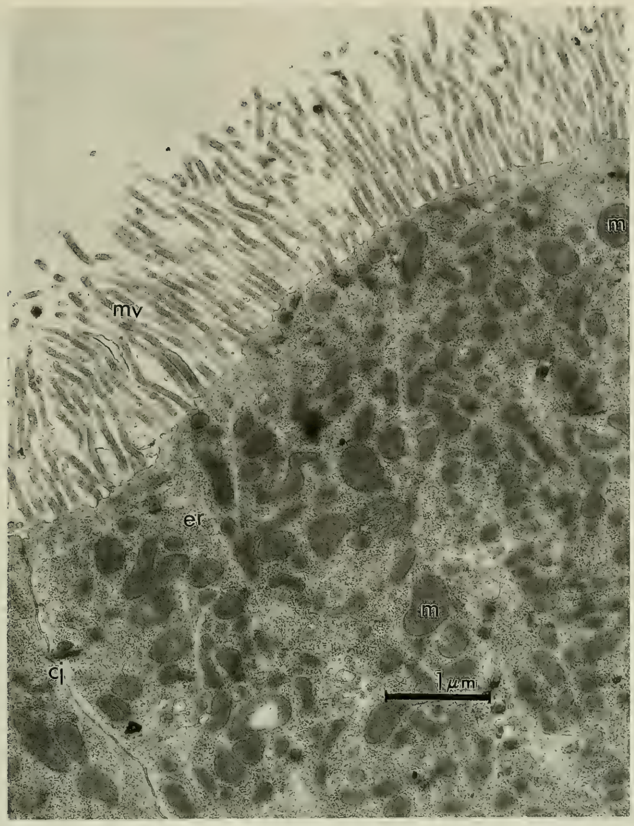


Figure 1. Transmission electron micrograph of the larval midintestine where β-galactosidase is produced. This micrograph shows the midintestinal epithelium of a larva cut in the transverse plane from a segment of larval midintestine that showed X-gal staining. No evidence of specialized cells containing encapsulated bacteria (mycetocytes) or bacteria was seen within the lumen. m, mitochondria; mv, microvilli; cj, cell junction; l, lumen of midintestine; er, endoplasmic reticulum.

racles, however, was lighter than in late third-instar larvae. Cryostat sections showed new areas of staining: loose, punctate clusters of stained cells distributed throughout the body but not directly associated with any organ, and a light, punctate staining in the anterior organs. As in late third-instar larvae, the wing and leg discs did not stain, nor did the brain, ventral ganglion, or fat body.

In prepupal stage P2, both whole mounts and sectioned material showed that the loose clusters of punctate staining increased in number; staining was also found in the developing eyes and antennae and was now found in the everted legs and wings as well (Fig. 6A, B). Clusters of stain appeared in cavities within the evaginated wing rudiments and were found internally at the proximal end of developing legs. The anterior region housing the imaginal discs had areas of foamy-appearing tissue, rich in droplets, probably of lipid, surrounded by diffuse stain. The lymph glands (Fig. 6C) continued to stain intensely, and the midintestine, which at this stage is in the process of being remodeled, now stained more darkly. The brain and ventral ganglion were beginning to change shape from the larval form but did not stain, nor did the fat body.

In prepupal stage P3, the staining patterns evident in P2 persisted and intensified. The punctate clusters of stain became more abundant and were distributed throughout the body. At this stage the abdominal pericardial cells began to show dark staining (Fig. 6D). The developing brain and ventral ganglion did not stain.

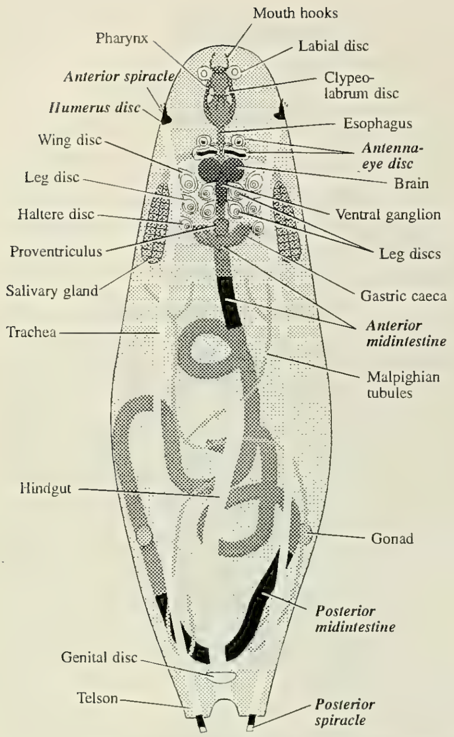

Mouth hooks
Pharynx
Labial disc
Anterior spiracle
Clypeo-labrum disc
Humerus disc
Esophagus
Wing disc
Antenna-eye disc
Brain
Leg disc
Ventral ganglion
Haltere disc
Leg discs
Proventriculus
Salivary gland
Gastric caeca
Anterior midintestine
Trachea
Malpighian tubules
Hindgut
Gonad
Posterior midintestine
Genital disc
Telson
Posterior spiracle

Figure 2. Diagram of endogenous β-galactosidase activity in the late third-instar larva stained at pH 7.2. After X-gal staining, β-galactosidase activity (black) is evident in the anterior and posterior spiracles, distinct segments of both the anterior and posterior midintestine, cellular epidermis, lymph glands, and eye-antenna imaginal discs. (Anatomy after Bodenstein, 1965; Tyler, 1994.)

In the prepupal stage P4, the amount of foamy-appearing tissue with diffuse stain increased, and stain was now found throughout the developing intestinal system. The fat body showed light staining. Again, there was no staining in the brain or ventral ganglion.

Pupal stages (P5–P15). During metamorphosis, the delicate nature of the remodeling tissue disallowed whole-mount dissections, but X-gal staining of cryostat serial sections revealed dramatic changes in staining intensity and distribution (Figs. 6 and 7).

After head eversion, P5, and in stage P6, there was an increase in dark, punctate staining which co-localized with the fat-body tissue of the head capsule, thorax, and abdomen. By stage P6, dark staining was associated with the first pupal spiracles and trachea. The staining associated with foamy-appearing tissues was no longer found in the head region but persisted in the developing legs

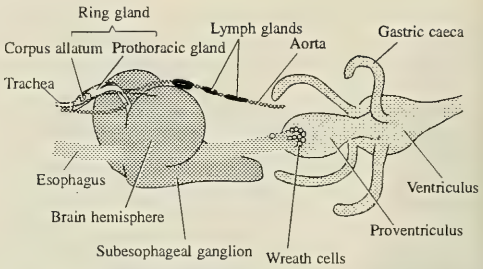

Ring gland
Corpus allatum
Prothoracic gland
Lymph glands
Aorta
Gastric caeca
Trachea
Esophagus
Ventriculus
Brain hemisphere
Proventriculus
Subesophageal ganglion
Wreath cells

Figure 3. Diagram of late third-instar larval nervous system and lymph glands. X-gal staining at pH 7.2 of β-galactosidase activity (black) is found in three pairs of larval lymph glands flanking the aorta. Light X-gal staining was seen in the lymph glands of some active larvae. Pronounced staining of these tissues was apparent in all of the inactive larvae sampled. Anterior to left, dorsal is up. (Anatomy after King, 1970.)

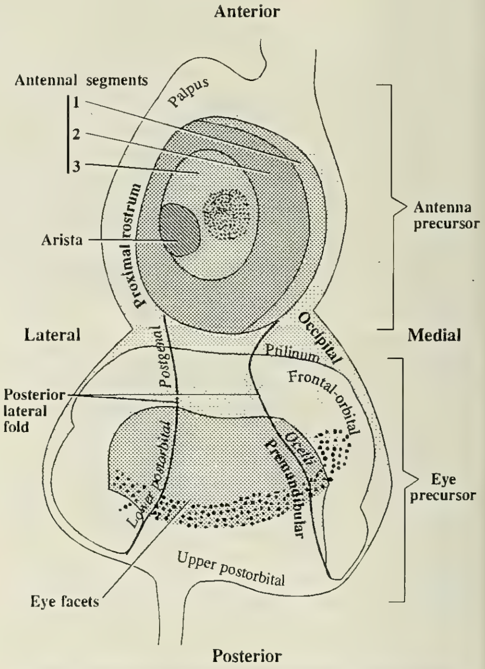

Anterior
Antennal segments
1
2
3
Palpus
Antenna precursor
Arista
Proximal rostrum
Lateral
Medial
Occipital
Ptilinum
Postgenal
Posterior lateral fold
Frontal-orbital
Ocelli
Lower postorbital
Premandibular
Eye precursor
Upper postorbital
Eye facets
Posterior

Figure 4. Diagram of endogenous β-galactosidase activity in the eye-antenna imaginal disc stained at pH 7.2. After X-gal staining, distinct patterns of β-galactosidase activity were revealed in all inactive larval and white prepupal (P1) discs. Punctate staining was localized to the center of the antenna precursor, and a horizontal band of stain was seen in the eye precursor. (Anatomy after Haynie and Bryant, 1986.)

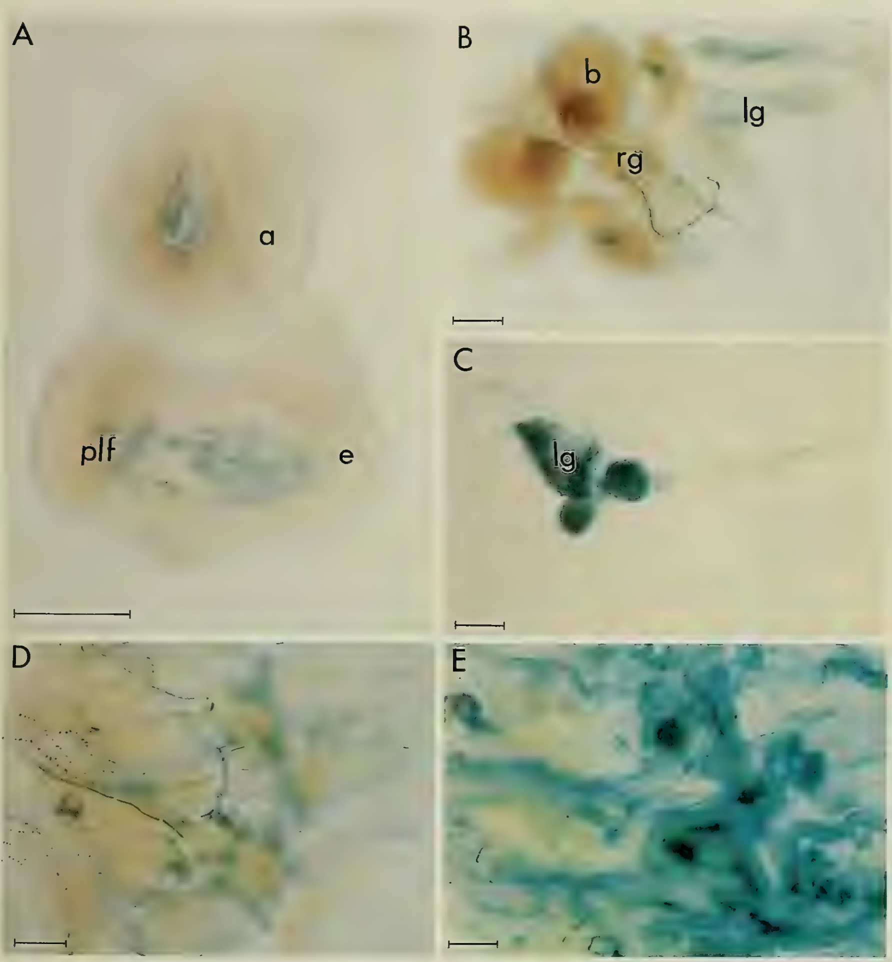


Figure 5. X-gal staining at pH 7.2 of β-galactosidase activity in eye-antenna imaginal discs, lymph glands, and cellular epidermis of active and inactive late third-instar larvae. A noticeable increase in β-galactosidase activity occurs in these tissues as the larva becomes inactive. Bars = 0.1 mm. (A) Whole mount of eye-antenna disc from an inactive larva. X-gal staining intensifies and is found in distinct clusters of cells in both the antennal and eye precursors. a, antennal precursor; e, eye precursor; plf, posterior lateral fold. (B) Whole mount of the brain and lymph glands of an active larva. Light staining is present in the lymph glands. lg, lymph gland; b, brain; rg, ring gland. (C) Whole mount of inactive larval lymph glands. As the larva becomes inactive, the lymph glands become mottled with patches of dark stain. lg, lymph gland tissue. (D) Whole mount of active larval cuticle and cellular epidermis. The cellular epidermis is lightly stained. (E) Whole mount of inactive larval cuticle and cellular epidermis. Staining of the cellular epidermis intensifies and appears to be concentrated in cell clusters.

and wings. The relatively compact tissues of the developing digestive system showed little staining. The brain and ventral ganglion did not stain.

The highest intensity of staining was apparent in stages P7 and P8 (Fig. 6E). Dark, punctate staining was distributed throughout the fat body, and a concentrated "ball"

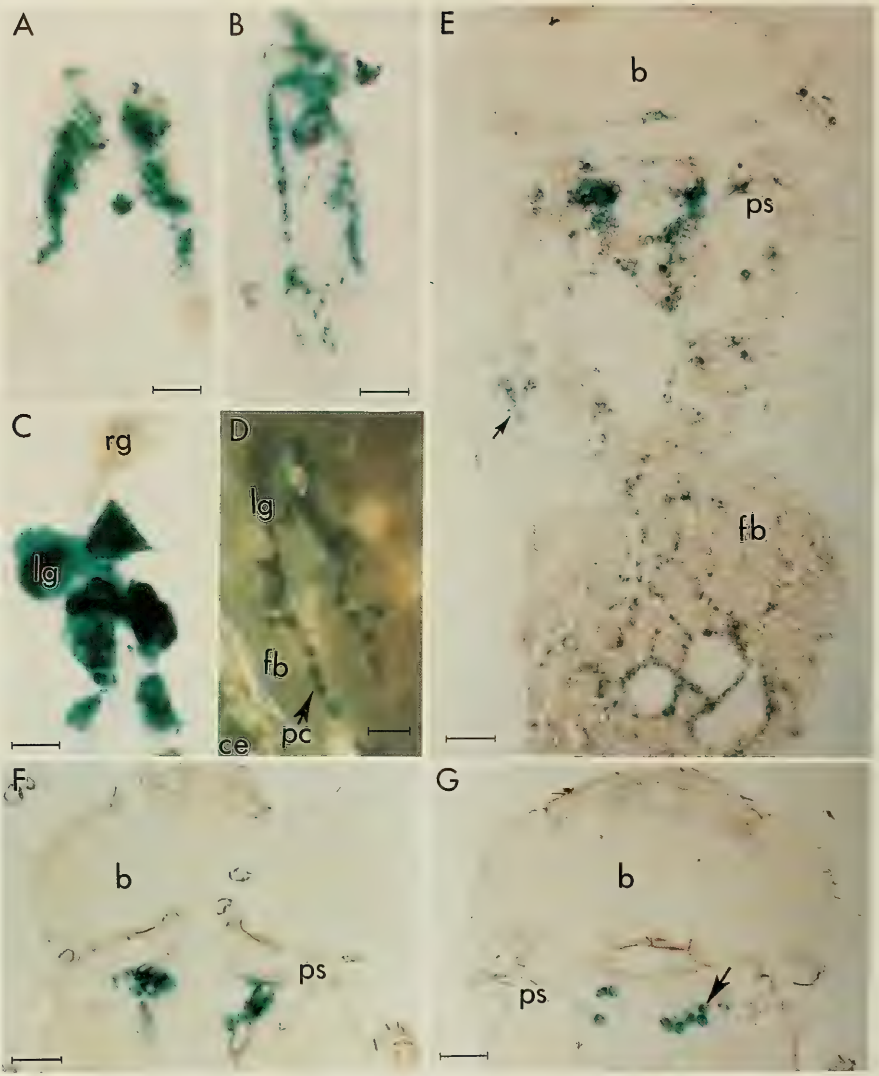


Figure 6. X-gal staining at pH 7.2 of β-galactosidase activity in pupal appendages, lymph glands, and pericardial cells. Bars = 0.1 mm. (A,B) Whole mounts of evaginated discs from pupal stage P2. In the leg discs (A), intense staining is found internally at the proximal end of the appendage, a compartment that is continuous with the haemocoel. In developing wing rudiments (B), intense punctate staining is found in narrow cavities running down the length of the wing in areas that eventually give rise to the wing veins. (C) Whole mount of stage P2 lymph glands. When compared to the larval lymph glands (Fig. 4C), staining intensity clearly increases in these tissues during the pupal stage. Anterior is up. lg, lymph glands; rg, ring glands. (D) P3 pupa after dissection and staining. Punctate staining appears throughout the body cavity and in cellular epidermis region, developing appendages, and gut (removed from this preparation). The pericardial cells, posterior to the lymph glands and flanking the aorta and heart, show intense staining. Anterior is up. lg, lymph glands; ce, cellular epidermis; fb, fat body; pc, pericardial cells. (E) Cryostat sections of pupal stage P7. Intense punctate staining occurs throughout the head, thoracic and abdominal fat-body tissue. Staining is most intense at the first pupal spiracle. Some β-galactosidase activity is still present in the developing appendages (arrow). Stages P7 and P8 exhibit the highest levels of enzyme activity found through metamorphosis. Anterior is up. b, brain; fb, fat body; ps, pupal spiracle. (F, G) Longitudinal cryostat sections of pupal stage P12 (F) and P13 (G) after staining. Intense staining is found medial to the first pupal spiracle and, in P13 (G), clearly appears in clusters (arrow) that may be the developing thoracic nephrocytes. Anterior is up. b, brain; ; ps, first pupal spiracle.

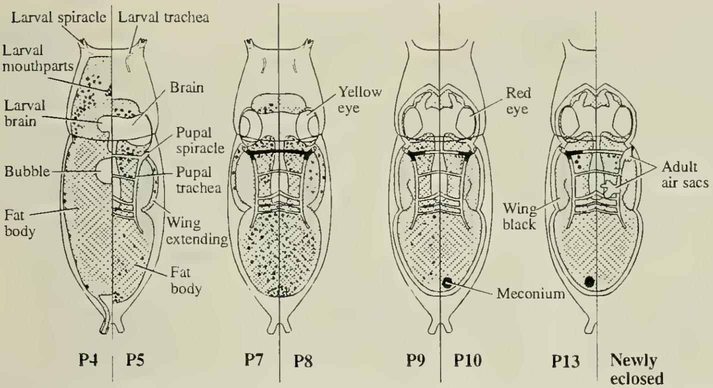


Figure 7. Schematic summary through metamorphosis of endogenous β-galactosidase activity stained at pH 7.2. Based on X-gal-stained 20-μm serial frozen longitudinal sections of pupal stages 1–15 cut in the frontal plane. At stage P4, staining (black) is primarily localized to the region housing the imaginal discs, remodeling midgut, and cellular epidermis. After head eversion, P5, there is light staining of the head, thoracic and abdominal fat-body tissue, with darker staining associated with the pupal spiracle. Developing legs and wings exhibit light diffuse staining of foamy-appearing tissue characteristic of the imaginal tissues. In stages P7 and P8, punctate staining intensifies in thoracic and abdominal fat body. Dark staining appears in the first dorsal anastomosis between the two spiracles. Lighter staining persists in developing legs and wings. A concentrated "ball" of stain is seen at the tip of the abdomen. Staining decreases dramatically at stage P9. In stages P10–P14, dark staining is only associated with the pupal spiracle region and in a region where the meconium is found. The fat body and developing digestive system show low levels of staining. (Anatomy after Bainbridge and Bownes, 1981; tracheal anatomy after Whitten, 1980.)

of stain appeared at the tip of the abdomen. As with the earlier stages, there was little or no staining of compact tissues. The staining associated with the developing appendages, pupal spiracle, and trachea persisted.

In stage P9, the stain in the thoracic and abdominal fat body decreased and remained at low levels through P14. At stage P10 and in subsequent pupal stages, no stain was found in the developing appendages.

Through stages P9–P14, intense stain was associated with the anterior spiracle region, along the line of the first dorsal anastomosis and into the dorsal tracheal trunks (Fig. 6F) (anatomy after Whitten, 1980). In pupal stages P12–P15, staining at the spiracle was not confined to the tracheal tissues but also appeared as clusters of stain in the tissue surrounding the trachea (Fig. 6G). These clusters of stain appeared to be the developing thoracic nephrocytes, which stain in the adult. Punctate staining appeared between the muscle masses forming in the dorsal thorax in the region where air sacs are developing. A patch of dark stain also consistently appeared in stages P12–P15 at the dorsal tip of the abdomen in a region where the meconium is found. The meconium, which contains the remains of the larval intestinal epithelium, gastric caecae (Bodenstein, 1965), and other by-products of metamorphosis such as excess hormones (Bownes, 1990), is excreted after eclosion.

Adults. In dissected adult flies, intense X-gal staining was found in the thoracic nephrocytes (Fig. 8A), abdominal pericardial cells (Fig. 8B), and two clearly demarcated regions of the ventriculus (Fig. 8C). Staining was also consistently found in the reproductive systems of male and female flies aged 1 to 2 days, 3 to 5 days, and 7 to 10 days. There was staining in the ejaculatory pump of 1- to 2-day-old male flies (Fig. 8D), which intensified in the older 3- to 5- and 7- to 10-day-olds (Figs. 8C, 9). In the female, small clusters of stain were found in the calyx and pedicel of the ovary (Figs. 8E, 9), and there was dark staining of the uterus (Figs. 8F, 9). In newly eclosed flies, punctate staining was found associated with the fat body and there was diffuse light staining of the rectal papillae. In the reproductive systems of immature females, staining was found only in the uterus.

Staining of endogenous β-galactosidase activity at pH 6.0

Third instar larval and prepupal stages (Table I). In the third-instar larval and prepupal stages, the staining patterns at pH 6.0 differed from those at pH 7.2 primarily in the intensity of staining. Areas that stained at pH 7.2 (cellular epidermis, eye-antenna disc, everting legs and wings, lymph glands, midintestine, larval spiracles, internal punctate

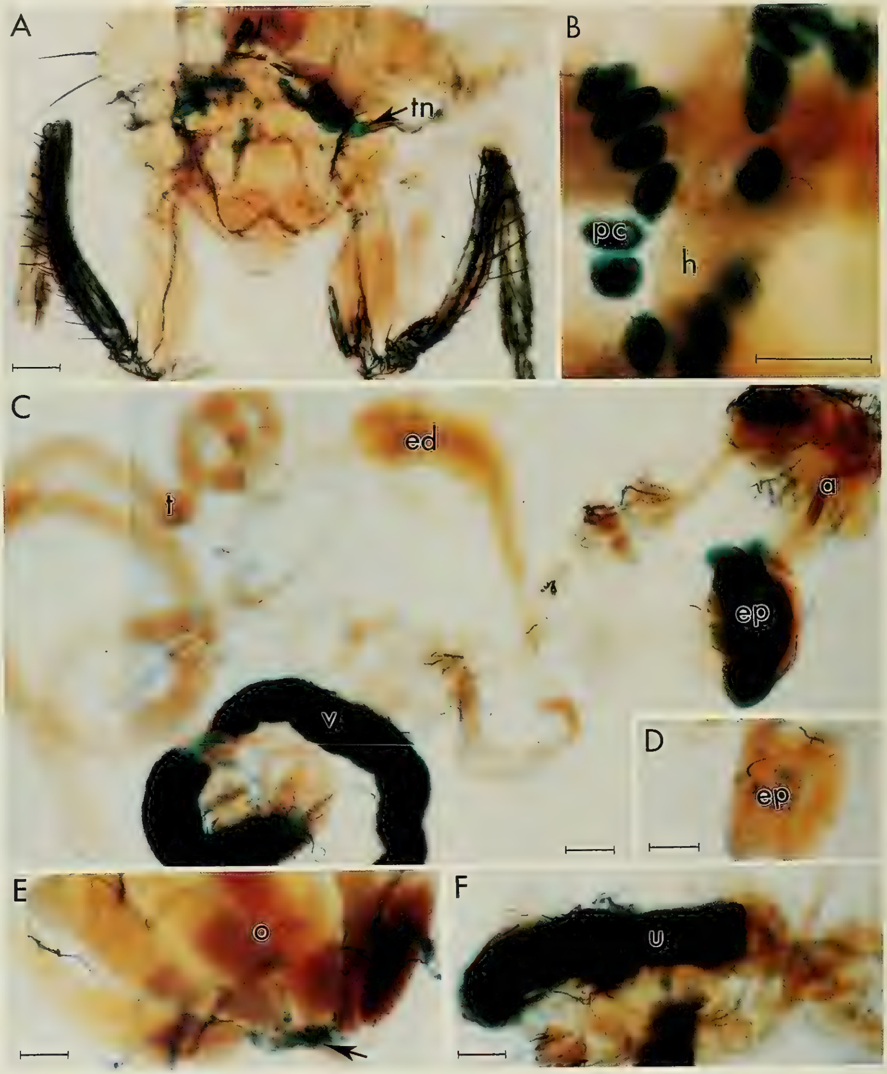
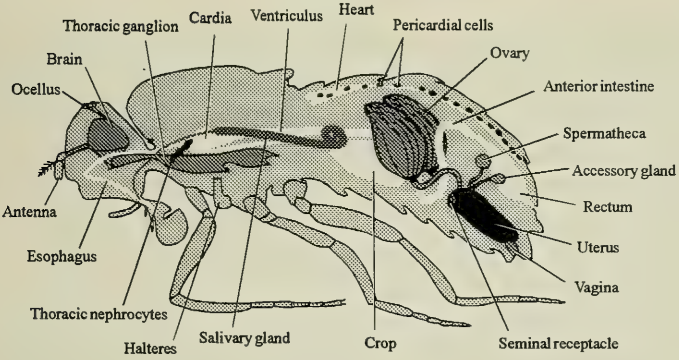
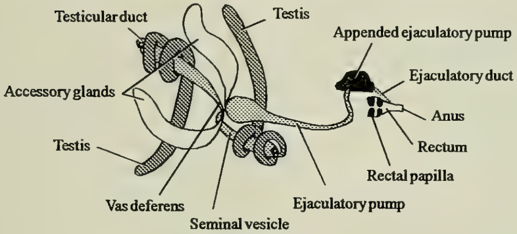


Figure 8. X-gal staining at pH 7.2 of β-galactosidase activity in the adult pericardial cells, thoracic nephrocytes, reproductive and digestive systems. Bars = 0.1 mm. (A) Whole mount of adult fly with head removed to show stained thoracic nephrocytes. This staining was also seen in all adults sampled, regardless of age. tn, thoracic nephrocytes. Dorsal is up. (B) The abdominal pericardial cells flanking the heart of adult flies (of all ages) showed intense staining. pc, pericardial cell; h, heart. (C) Whole mount of reproductive system and lower digestive system from a 3–5-day-old male. Intense staining is found in the ejaculatory pump and in two regions of the ventriculus. (D) Whole mount of ejaculatory pump from a 1–2-day-old male has low levels of enzyme activity. No staining was found elsewhere, including the ejaculatory duct, testes, or rectum. a, anus; ed, ejaculatory duct; ep, ejaculatory pump; r, rectum; t, testes. (E) Whole mount of the female reproductive system from a 3–5-day-old fly shows β-galactosidase activity in the calyx and pedicel of the ovary (arrow) and in the uterus (F). co, common oviduct; o, ovary; u, uterus.


Thoracic ganglion
Cardia
Ventriculus
Heart
Pericardial cells
Ovary
Brain
Ocellus
Anterior intestine
Spermatheca
Accessory gland
Antenna
Rectum
Esophagus
Uterus
Vagina
Thoracic nephrocytes
Halteres
Salivary gland
Crop
Seminal receptacle

Female


Testicular duct
Testis
Appended ejaculatory pump
Ejaculatory duct
Accessory glands
Anus
Testis
Rectum
Rectal papilla
Vas deferens
Ejaculatory pump
Seminal vesicle

Male reproductive system

Figure 9. Diagram of endogenous β-galactosidase activity at pH 7.2 in adult female and male flies. After X-gal staining, β-galactosidase (black) was found in the thoracic nephrocytes, abdominal pericardial cells, and ventriculus (not shown in this diagram) of all adults, regardless of age. In females, staining in the uterus was seen after eclosion, but no stain was seen in the calyx and pedicel of the ovaries until the flies were 1–2 days old. In the male reproductive system, light staining was seen in the sperm pump of 1–2-day-old flies and intensified as the fly aged. Light staining was seen in the rectal papillae of newly eclosed flies. (Anatomy after Miller, 1965.)

staining regions, and pericardial cells) stained more intensely at pH 6.0. The pericardial cells stained at an earlier stage at pH 6.0 than at 7.2. Some structures that stained at pH 6.0 did not stain at pH 7.2: the garland (wreath) cells, which stained darkly, and the proventriculus, ventral ganglion, and testes—all of which stained lightly.

At pH 6.0, as at pH 7.2, overall staining levels increased as the animals approached pupation.

Pupal stages (Table II). In the pupa, as in the prepupal stages, the primary difference in staining patterns between specimens stained at the two pH levels was in intensity of staining. Areas that stained at pH 7.2 (fat body, developing legs and wings, first pupal spiracle, tracheae, punctate staining between dorsal thoracic muscles, and developing digestive system) stained more intensely at pH 6.0. Staining persisted longer in developing legs and wings at pH 6.0 than at pH 7.2. Some structures in cryostat sections stained at pH 6.0 but not at pH 7.2: brain and ventral ganglion and antennae.

As at pH 7.2, the intensity of staining increased with increased age between stages P4 and P8. The intensity appeared to peak at P7–P9.

Adult stages (Table III). In the adult, as in the other stages, a major difference between staining patterns at pH 7.2 and 6.0 was in intensity, with areas that stained at pH 7.2 staining more intensely at pH 6.0 (ventriculus, fat body, calyx and pedicel, and sperm pump). Some regions of staining did not change in intensity (thoracic nephrocytes and pericardial cells). Some structures that stained at pH 6.0 did not stain at pH 7.2: testes, antennae and brain and, in the newly eclosed flies, the thoracic ganglion and halteres. The staining in the brain was pronounced

Table I

Areas of X-gal staining for endogenous β-galactosidase activity in third-instar larval and prepupal stages of Drosophila melanogaster *in staining buffers at pH 7.2 and 6.0*

Larval stage	Larval cellular epidermis pH 7.2/6.0	Eye-antenna disc pH 7.2/6.0	Leg & wing discs pH 7.2/6.0	Lymph glands pH 7.2/6.0	Mid-intestine pH 7.2/6.0	Larval spiracles pH 7.2/6.0	Internal punctate staining pH 7.2/6.0	Pericardial cells pH 7.2/6.0	Garland cells pH 7.2/6.0	Proven-triculus pH 7.2/6.0	Ventral ganglion pH 7.2/6.0	Testes pH 7.2/6.0
Active larva	+/+*	+[1]/+[1]	−/−	+/+	+/+*	+/+	−/−	−/+	−/+	−/−	−/−	−/+
Inactive larva	+/+*	+/+*	−/−	+/+*	+/+*	+/+*	−/−	−/+	−/+	−/+	−/+	−/+
P1	+/+*	+/+*	−/−	+/+*	+/+*	+/+*	+/+*	−/+	−/+	−/+	−/+	−/+
P2	+/+*	+/+*	+/+*	+/+*	+/+*	+/+*	+/+*	−/+	−/+	−/+	−/+	−/+
P3	+/+*	+/+*	+/+*	+/+*	+/+*	−/−	+/+*	+/+*	−/+	−/+	−/+	−/+

−, no staining; +, staining; +*, staining intensified over pH 7.2 levels.

[1] Occasionally stained.

in newly eclosed flies, including areas of the cellular cortex, cell body layer of the lamina, and the first and second optic chiasmata (Fig. 10). In adults aged 1–2 days and older, staining persisted only in the cell-body layer of the lamina. No differences in these staining patterns were noted between males and females.

Discussion

We have characterized tissue-specific activity of endogenous β-galactosidase in wild-type *Drosophila melanogaster* by staining with the chromogen X-gal at pH 7.2 and 6.0. Our findings show that at both pH levels, β-galactosidase activity is found in a variety of tissues in the larval, pupal, and adult stages and that striking changes in tissue-specific activity of the enzyme occur at the larval-pupal transition and through metamorphosis. The staining patterns shown at pH 7.2 are particularly germane for studies in which X-gal is used to assay *lacZ* fusion gene or enhancer trap activity in *D. melanogaster.* At the staining pH typically recommended for these studies (pH 7.2; Ashburner, 1989), animals will not only exhibit endogenous enzyme activity in certain tissues throughout developmental stages, but will also exhibit sites of activity that change within a single stage.

In our study, we discovered certain tissue-specific activities that have not previously been reported. At staining pH levels of 7.2 and 6.0, sites of activity were the lymph glands, cellular epidermis, and eye-antenna imaginal discs of the larva and the thoracic nephrocytes and fat body of the adult. At a staining pH of 6.0, additional sites were the larval garland (wreath) cells, proventriculus, ventral ganglion and gonads; the pupal brain, ventral ganglion, and antennae; and the adult brain and antennae, with the halteres and thoracic ganglion staining in the newly eclosed fly. These results, coupled with the fact that staining at pH 6.0 in general was more intense than at pH 7.2, show that staining patterns are pH-dependent.

A previous report of endogenous β-galactosidase activity in the larval midintestine, adult ventriculus, pericardial cells, and ejaculatory pump (Glaser *et al.,* 1986) was based on cultures that were not grown axenically. Because our axenic cultures showed the same activity, we conclude that these regions of β-galactosidase activity are not due to the presence of commensal flora in the fly. In limited studies of nonaxenic cultures, we did, however, find instances of variable X-gal staining that we did not find in the axenic cultures; for example, in the adult (3-day and older) appendages and rectum (unpubl. data). This suggests that bacterial contamination can contribute to the X-gal staining reaction, and that it may be wise to grow flies axenically to eliminate this variable.

Our results should not preclude the use of transformed flies in a *lacZ*/X-gal assay, but the pH of the staining buffer should be carefully monitored and the flies should be staged precisely. If staging errors are made—for example, if an active third-instar larva is used as a control animal but a slightly older inactive transformed larva is assayed for *lacZ* fusion-gene activity—the pronounced endogenous activity in the eye and antenna imaginal discs, lymph glands, and cellular epidermis could be construed as ectopic expression of the *lacZ* fusion-gene product. In some reports from studies on *D. melanogaster* using *lacZ* fusion gene transformants, X-gal staining in the eye and antenna discs of third-instar larvae was attributed to the fusion-gene product, with no mention of any similar staining patterns in the controls (*e.g.,* Glaser *et al.,* 1986; Brand and Perrimon, 1993). In light of our evidence for the need to control for age-dependent expression within the same life stage, such results should be reexamined with attention to the staging of the larvae. In addition, the finding that, during metamorphosis, the endogenous enzyme activity is expressed at high levels in many tissues—such as

Table II

Areas of X-gal staining for endogenous β-galactosidase activity in cryostat sections of pupal stages of Drosophila melanogaster *in staining buffers at pH 7.2 and 6.0*

Pupal stage	Fat body pH 7.2/6.0	Developing legs & wings pH 7.2/6.0	Developing digestive system pH 7.2/6.0	First pupal spiracle pH 7.2/6.0	Tracheae pH 7.2/6.0	Brain & ventral ganglion pH 7.2/6.0	Developing antennae pH 7.2/6.0
P4	+/+*	+/+	+/+*	−/−	−/−	−/+	−/−
P5	+/+*	+/+	+/+*	+/+*	−/−	−/+	−/−
P6	+/+*	+/+	+/+*	+/+*	+/+*	−/+	−/−
P7–8	+/+*	+/+	+/+*	+/+*	+/+*	−/+	−/−
P9	+/+*	+/+*	+/+*	+/+*	+/+*	−/+	−/+
P10–14	+/+*	−/+	+/+*	+/+*	+/+*	−/+	−/+

−, no staining; +, staining; +*, staining intensified over pH 7.2 levels.

N.B. No data were collected on staining patterns of certain structures that are poorly defined in cryostat sections of pupal stages. These include the gonads, pericardial cells and garland cells.

the developing legs, wings, alimentary tract, and fat body—even at a staining pH (7.2) that is meant to exclude endogenous activity, use of an anti-*E. coli* β-galactosidase antibody, a different reporter gene (such as luciferase), or a different molecular assay would be appropriate when investigating gene activity in these tissues.

The presence of β-galactosidase activity in several organ systems and throughout metamorphosis suggests that this enzyme might be involved in several vital physiological processes in *D. melanogaster.* The increases in the distribution and level of enzyme activity before and during metamorphosis are particularly interesting, because they coincide with known peaks of ecdysteroid titer (Bainbridge and Bownes, 1988; Pak and Gilbert, 1987; and Handler, 1982). The appearance of endogenous β-galactosidase activity in the ventral ganglion, eye and antenna imaginal discs, lymph glands, and cellular epidermis in late third-instar larvae just prior to pupariation coincides with a dramatic increase in ecdysteroid titer 10 h before puparium formation (Bainbridge and Bownes, 1988). Additionally, the increase in β-galactosidase activity in pupal stages P5–P9, peaking at stages P7 and P8, coincides with a high concentration of ecdysteroids that peaks at P7 (Bainbridge and Bownes, 1988). The possibility of a relationship between ecdysterone and β-galactosidase expression is further supported by the observation that ecdysterone induces the expression of β-galactosidase in *D. melanogaster* cell cultures (Best-Belpomme *et al.,* 1978).

The physiological role of β-galactosidase in *D. melanogaster* has not been characterized. If the β-galactosidase in *D. melanogaster* is involved in activities akin to those of a mammalian lysosomal form (for example, see Wallenfels and Weil, 1972; Belfiore, 1980), the induction and expression of the enzyme during metamorphosis is not surprising, because enzymatic degradation of macromolecules is widespread during this period. During metamorphosis, larval cells undergo histolysis and are attacked by phagocytic cells. These phagocytic cells degrade the debris from the larval cells, and material is recycled for

Table III

Areas of X-gal staining for endogenous β-galactosidase activity in adult stages of Drosophila melanogaster *in staining buffers at pH 7.2 and 6.0*

Adult stage	Thoracic nephrocytes & pericardial cells pH 7.2/6.0	Ventriculus pH 7.2/6.0	Fat body (punctate staining) pH 7.2/6.0	Calyx & pedicel pH 7.2/6.0	Uterus pH 7.2/6.0	Sperm pump pH 7.2/6.0	Testes pH 7.2/6.0	Rectal papillae pH 7.2/6.0	Antennae pH 7.2/6.0	Thoracic ganglion pH 7.2/6.0	Halteres pH 7.2/6.0	Brain pH 7.2/6.0
Newly eclosed	+/+	+/+*	+/+*	−/−	+/+*	−/+	−/−	+/+	−/+	−/+	−/+	−/+
1–2 Day	+/+	+/+*	−/−	+/+*	+/+*	+/+*	−/+	−/+	−/+	−/−	−/−	−/+
3–5 Day	+/+	+/+*	−/−	+/+*	+/+*	+/+*	−/+	−/−	−/−	−/−	−/−	−/+
7–10 Day	+/+	+/+*	−/−	+/+*	+/+*	+/+*	−/+	−/−	−/−	−/−	−/−	−/+

−, no staining; +, staining; +*, staining intensified over pH 7.2 levels.

[1] Occasionally stained.

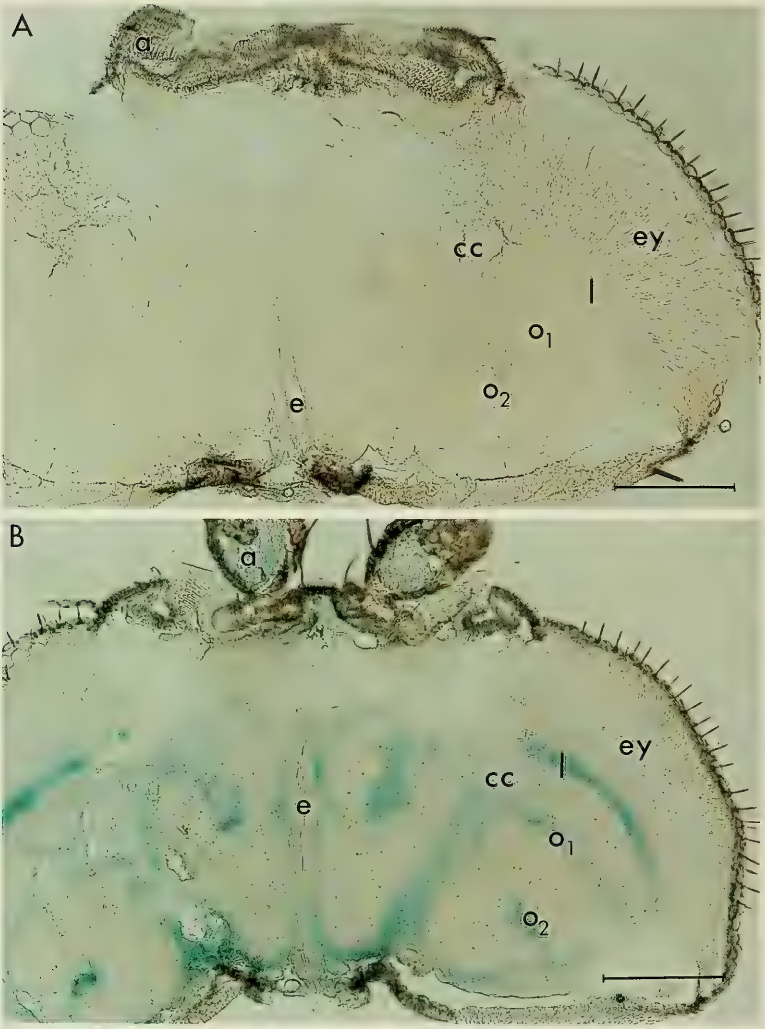


Figure 10. X-gal staining of β-galactosidase activity at pH 7.2 (A) and pH 6.0 (B) of cryostat frontal sections of adult heads. Anterior is up. a, antenna; cc, cellular cortex; e, esophagus; ey, eye; l, cell body layer of the lamina; o_1, first optic chiasmata; o_2, second optic chiasmata. Bars = 0.1 mm. (A) Section through head of a newly eclosed female stained at pH 7.2. No staining shows up in the brain. (B) Alternate section of the above, stained at pH 6.0. Staining occurs in numerous regions of the brain, including the first and second optic chiasmata, cellular cortex, and cell body layer of the lamina. Light staining is also seen in the antennae.

use by the developing imaginal tissues (Chapman, 1969). Many of the areas that we found to have β-galactosidase activity are associated with phagocytic activity: the lymph glands release large numbers of phagocytic cells into the haemocoel through the prepupal stage (Rizki, 1978a); in the eye and antenna discs, localized programmed cell death, correlated with high concentrations of lysosomes (Murphy, 1974), occurs during morphogenesis (Nöthiger, 1972; Poodry, 1980; Kankel *et al.*, 1980); the protein globules of the fat body are thought to be cytolysosomal or autophagic bodies (Rizki, 1978b) that are discharged from the cells into the intercellular space during pupation (Yagi, 1962); the garland cells, thoracic nephrocytes, and pericardial cells, considered stationary macrophages, engulf microbes and remove toxins from the haemolymph—a role analogous to that of the vertebrate reticuloendothelial system (Wigglesworth, 1972). To determine whether the β-galactosidase activity that we see is found in lysosomal compartments, we plan to examine the subcellular localization of the enzyme in the lymph glands, thoracic nephrocytes, and pericardial cells.

The physiological significance of invertebrate β-galactosidases is unexplored. In light of our findings, *D. melanogaster* appears to be an appropriate organism for such an investigation.

Acknowledgments

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Mortality of Pentactulae During Intraovarian Brooding in the Apodid Sea Cucumber *Leptosynapta clarki*

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Abstract. Estimates of mortality during development in brooders have been restricted by the lack of a reliable method for determining the number of embryos at the start of brooding. The calcareous ring of the pentactulae of *Leptosynapta clarki* can be used to determine initial embryo number because the ring is present for about 93% of the brooding period, is retained within the ovary, and is not resorbed after embryo death. Pentactulae mortality was found to be variable in female *Leptosynapta clarki,* with up to 100% mortality (= total brood loss) in some females. Estimates of instantaneous mortality in *L. clarki* ranged from 0 to $-0.0114\ \mathrm{d}^{-1}$ and were of similar magnitude to those for other brooding marine invertebrates. The resorption of dead pentactulae in the ovary may provide additional material to the ovarian fluid for the nutrition of living pentactulae. The retention of hard embryonic structures (*e.g.,* calcareous parts, shells) in other brooding marine invertebrates may allow further estimates of mortality during brooding, and provide an insight into the importance of embryo mortality in the evolution of marine invertebrate life histories.

Introduction

Determination of natural mortality rates in marine invertebrate larvae has presented a considerable challenge to scientists for many years (Rumrill, 1990). Although there are many estimates of larval mortality in planktonic species (see reviews: Chaffee and Strathmann, 1984; Strathmann, 1985; Hines, 1986; Rumrill, 1990), few studies quantify mortality during encapsulation or brooding (but see Rumrill, 1990; Rawlings, 1990). The authors of these and other studies (*e.g.,* Grant, 1983) have noted the need for estimates of mortality in nonpelagic species, and the importance of estimating "the relationship between the energy put into brood protection and the reduction in mortality which results" (Grant, 1983, p. 554).

Measurements of embryo mortality during benthic development have been limited by the need to know the initial number of embryos (N_o) that began the developmental period. Such values are particularly difficult to obtain in brooding species because there is usually no method for determining N_o without removing the brood from the female. Such an invasive technique would preclude any further study of the brood success of that female. Further, as the reproductive season progresses it becomes more difficult to determine a value for N_o in an individual female without confounding values for initial embryo number with subsequent mortality.

Estimates of embryonic mortality during brooding, although few, have been made in asteroids (Menge, 1974; Hendler and Franz, 1982; Strathmann *et al.,* 1984) and ophiuroids (Rumrill, 1982), and in holothuroids prior to hatching (Rutherford, 1973). These studies have, however, depended on inappropriate assumptions about fertilization success to determine the number of embryos (N_o) that began the developmental period. Menge (1974), for example, based his initial embryo number on the regression between egg number and female body size, assuming 100% fertilization. The assumption of complete fertilization success may not be valid in broadcast-spawning species (see review by Levitan 1995; Levitan and Petersen, 1995) and is generally untested in brooders (but see Sewell, 1994: 0%–98.5%, mean 27% in *Leptosynapta clarki*). If natural fertilization is less than 100%, embryo survival will be underestimated and mortality will be overestimated (Rumrill, 1990). The remaining studies of embryonic

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mortality assume that the rate of fertilization is the same in each female, and estimate mortality based on the difference in the number of early- and late-stage embryos in different females (Hendler and Franz, 1982; Strathmann *et al.,* 1984; Rumrill, 1982) or consider only prehatching mortality (Rutherford, 1973). In the latter cases, assuming high fertilization success in females with early stages of development would also overestimate mortality by inflating the value of N_o.

This study reports an innovative approach for measuring embryonic mortality in the intraovarian brooding sea cucumber *Leptosynapta clarki.* This species broods pentactulae in the ovarian tubules (*i.e.,* is viviparous, Komatsu *et al.,* 1990; Wourms and Callard, 1992) from November to April or May (Everingham, 1961; Sewell and Chia, 1994). Development to the pentactula stage is rapid (about 10–14 days) and is synchronous within individual females (Sewell and Chia, 1994). Pentactulae, like juvenile and adult sea cucumbers, have a calcareous ring at the base of the tentacles (Everingham, 1961; Sewell and Chia, 1994). Although death of the pentactula results in the resorption of the embryonic tissues, the calcareous ring remains visible in the ovarian tubule. Counts can thus be made of the numbers of living and dead pentactulae, which in total give N_o, and the pentactulae mortality can be determined for each female. This paper reports mortality in *L. clarki* pentactulae throughout the brooding period and suggests that mortality in this viviparous species is variable, and can be extremely high in some females.

Materials and Methods

Adult *Leptosynapta clarki* were collected at two sites in Grappler Inlet, Bamfield, British Columbia, Canada (Site 1: 48°49′57″ N, 125°06′45″ W; Site 2: 48°50′ N, 125°06′36″ W). Site 1 is located on the northern side of Barge Bay, has a short intertidal area (*ca.* 20 m width) and a high proportion of gravel in the substrate. Site 2 is a gentler sloping mudflat (intertidal area *ca.* 60 m width) at the opposite end of the channel separating "No-name" Island from Vancouver Island proper (see Sewell and Chia, 1994, Fig. 1). This site has a finer sediment composition with little gravel and contains *L. clarki* of a larger size than Site 1 (Mean total weight ± SD, from Sewell and Chia, 1994: Site 1—307.8 ± 153.99 mg, $n = 720$; Site 2—490.4 ± 268.80 mg, $n = 660$). In the lower intertidal, both sites have extensive beds of *Zostera* sp. that extend into the shallow subtidal.

Specimens were collected from the mid-intertidal at each site, transported in buckets of mud to the laboratory, and kept overnight in running seawater. Sea cucumbers were screened out of the sediment with a 0.85-mm sieve and placed in a bowl of seawater. Female sea cucumbers that had not autotomized their posterior ends were chosen at random from the bowl and relaxed in 2.5% $MgCl_2$ in seawater (w/v) for 10–15 min. A relaxed sea cucumber was placed on a tissue to remove external water and weighed on a fine-scale balance to obtain total weight in milligrams. A longitudinal incision was then made from the base of the calcareous ring to the posterior, the coelomic water was drained from the body cavity, and the sea cucumber was reweighed to obtain its drained weight.

The ovary of *Leptosynapta clarki* is located in the anterior coelom and consists of two gonad tubules, one to each side of the dorsal mesentery (Sewell and Chia, 1994). The two gonad tubules were dissected from the coelom of each female under a binocular microscope, placed on a glass slide (38 × 75 mm) with a small amount of seawater, and temporarily mounted with a coverslip (35 × 50 × 0.02 mm). The weight of the coverslip revealed the pentactulae within the ovary without damaging the tubule. The slide was placed under a dissecting microscope and the numbers of living pentactulae, dead pentactulae, and calcareous rings were recorded for each female. This method may slightly underestimate total embryo mortality: firstly, a conservative attitude was adopted in counting a pentactula as dead; secondly, mortality estimates are for pentactulae only. Embryo mortality prior to this stage cannot be differentiated from resorbing unfertilized oocytes (see Discussion).

Samples of female *Leptosynapta clarki* were taken at Sites 1 and 2 at monthly intervals throughout the brooding period (November–May; Sewell and Chia, 1994). Ten females were dissected from each site at each time interval. Not all females were brooding pentactulae, however, so the number of females on which mortality is based varied between 1 and 10 at each site. In February 1991, mortality estimates were also included from females brooding pentactulae in gonad index dissections (Sewell and Chia, 1994). Photographs of ovaries with dead pentactulae were taken on a Leitz compound microscope immediately after counting was completed.

Relationships between percent mortality and features of the mother (female size = drained weight, number of pentactulae brooded) were tested by combining data from Sites 1 and 2 for each month. To reduce the probability of Type I error, the significance of the ordinary least squares (OLS) regressions was determined for each maternal feature by using a sequential Bonferroni test (Rice, 1989) and a groupwide Type I error of 0.05.

Results

Mortality estimates

Embryonic development in *Leptosynapta clarki* from Bamfield is as described for members of the species from False Bay, San Juan Island, Washington (Everingham, 1961). Hatching occurs after the prepentactula stage, producing a five-tentacled pentactula that has a calcareous ring. Pentactulae can move freely in the ovarian tubule (Everingham, 1961; Sewell and Chia, 1994). Development to the pentactula stage occurs in about 10–14 days, so embryos have a calcareous ring for approximately 93% of the 30-week brooding period (Sewell and Chia, 1994).

During dissections, dead pentactulae were observed in the ovarian tubules, identified by a discolored body wall (Fig. 1A), closed tentacles, incompletely connected gut (Fig. 1B), or contorted position. In advanced stages of resorption, the body of the pentactula was a "ghost" around the calcareous ring (Fig. 1C), or the calcareous ring was the only structure remaining in the ovarian tubule (Fig. 1D). By totaling the number of living pentactulae, dead pentactulae, and calcareous rings, the initial number of brooded pentactulae (N_o) could be determined and mortality calculated.

Mortality of pentactulae during brooding varied between females, ranging from 0% to 100%, with higher and more variable values during the last 3 months of brooding (Table I, Fig. 2). Differences were also observed in pentactulae mortality between both collection sites and years. Site 2 had a higher mean percent mortality in all samples in both years (Table I), and the mean percent mortality was higher in each month during 1990–1991 than in 1989–1990 (Table I, Fig. 2). Females generally brooded a lower number of pentactulae (Table I), and the number of females that had lost the entire brood was higher in the 1990–1991 reproductive season (1989–1990: 1 female; 1990–1991: 8 females; Fig. 2); a season that was marked by unusually cold conditions in December and January (days with temperatures below 0°C in December and January: 1989–1990 = 9 d; 1990–1991 = 25 d; P. Janitis, unpub. data).

Instantaneous mortality

Estimates of instantaneous mortality (day^{-1}) were calculated for pentactulae brooded in female *L. clarki* in March and April (1990, 1991) in the period prior to pentactulae release (Everingham, 1961; Sewell and Chia, 1994). The formula used was as defined in Strathmann (1985) and Rumrill (1990):

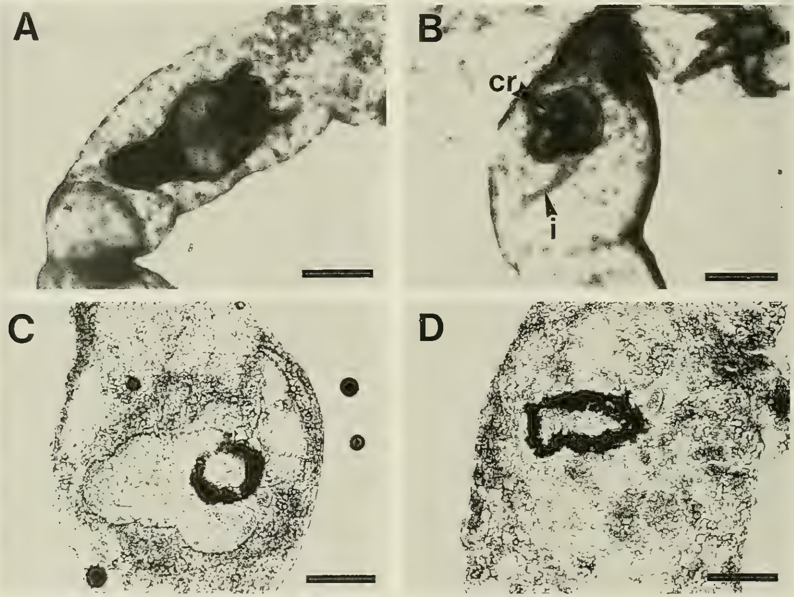

Figure 1. Photomicrographs of *Leptosynapta clarki* ovarian tubules during the brooding period. (A) Dead pentactula with discolored body wall. Note transparency of posterior of living pentactula in lower part of tubule. Bar = 500 μm. (B) Anterior end of dead pentactula showing calcareous ring (cr) at base of tentacles and resorbing intestine (i). Bar = 500 μm. (C) Dead pentactula with extensive resorption of the soft body tissues. Calcareous ring is visible within "ghost" of body. Bar = 500 μm. (D) Complete resorption of dead pentactula. Calcareous ring is the only remaining structure. Bar = 200 μm.

Table I

Percent mortality of brooded pentactulae and the total number of pentactulae (No.) brooded in female Leptosynapta clarki

	Percent mortality			No. pentactulae brooded			
Date	Mean	SD	Range	Mean	SD	Range	*n*
				Site 1			
1989–90							
16 Dec.	0	0		420	120.4	306–525	4
19 Jan.	1.3	1.1	0–2.7	302.3	210.0	2–783	10
9 Feb.	1.6	1.8	0–4.6	195.4	93.6	91–398	9
3 Mar.	10.5	12.7	0.7–37.0	202.4	176.1	61–588	10
3 Apr.	5.8	5.1	0–9.8	156.3	174.5	40–357	3
1990–91							
18 Nov.	0	—		—	—	245	1
24 Jan.	17.6	—		—	—	74	1
1 Feb.	57.4	38.1	9.4–100^{1}	52.2	34.4	19–107	6
25 Feb.	22.9	33.2	0–96.1	197.1	152.9	2–495	12
23 Mar.	9.5	8.6	0–29.4	65.7	37.1	2–128	11
2 Apr.	12.2	15.9	0–38.5	36.1	55.3	1–169	8
				Site 2			
1989–90							
23 Nov.	0	0		434.8	212.1	148–646	5
18 Dec.	4.3	4.7	0–13.0	273.8	307.3	23–839	9
21 Jan.	25.9	39.5	0–100^{1}	226.6	154.7	6–398	7
10 Feb.	15.1	20.8	0.5–62.9	146.2	85.9	39–299	9
4 Mar.	11.6	22.3	0–63.6	88.6	152.3	4–444	8
6 Apr.	40.1	33.0	0–76.5	19	21.6	2–56	5
1990–91							
24 Oct.	6.9	—		—	—	58	1
6 Dec.	0.3	—		—	—	319	1
17 Jan.	50.0	70.7	0–100^{1}	17.5	23.3	1–34	2
30 Jan.	59.9	46.8	11.3–100^{2}	91.5	38.0	53–141	4
27 Feb.	20.7	28.9	0–100^{1}	161.1	116.3	25–433	14
25 Mar.	38.0	17.8	16.4–66.2	41.8	23.1	14–67	6
1 Apr.	47.0	45.7	0.9–100^{3}	112.6	156.8	1–489	9

Sample size (*n*) refers to the number of brooding females sampled. Superscript values denote the number of females with 100% mortality in that sample.

$$M = \ln (N_o/N_t)/-t$$

assuming that fertilization occurred on 1 December of each year. This date was chosen because it falls in about the center of the spawning period for *L. clarki* at Bamfield (Sewell and Chia, 1994).

Values for instantaneous mortality in *L. clarki* are low (Table II) primarily because of the long brooding period (t = 93–128 days). Ten females had zero mortality (M = 0). Three females had lost their entire brood (*i.e.*, $N_o/N_t = 0$) and the value of M could not be calculated. For comparison to other species the grand mean for females with some living pentactulae is -0.0021 day^{-1} (SD = 0.003, n = 57).

Mortality in relation to features of the mother

No clear relationships were seen between the percent mortality and features of the mother such as size (drained weight) or number of pentactulae brooded (Table III). The relationship between percent mortality and the size of the female was nonsignificant for all sampling dates (Table III). The relationship between percent mortality and the total number of pentactulae brooded was significant in February 1991 only (Table III, Fig. 3). In this month, females brooding the fewest pentactulae tended to have the highest percent mortality (Fig. 3).

There was no maternal feature that consistently separated females with extremely high mortality from other females. For example, females with total brood loss (100% mortality) were within the same general size range as those of females with < 100% mortality (mean ± SD mg total weight: 100% mortality—372 ± 180, n = 9; < 100% mortality—368 ± 184, n = 146; t = 0.063, NS). However, females with 100% mortality were brooding fewer pentactulae (mean ±

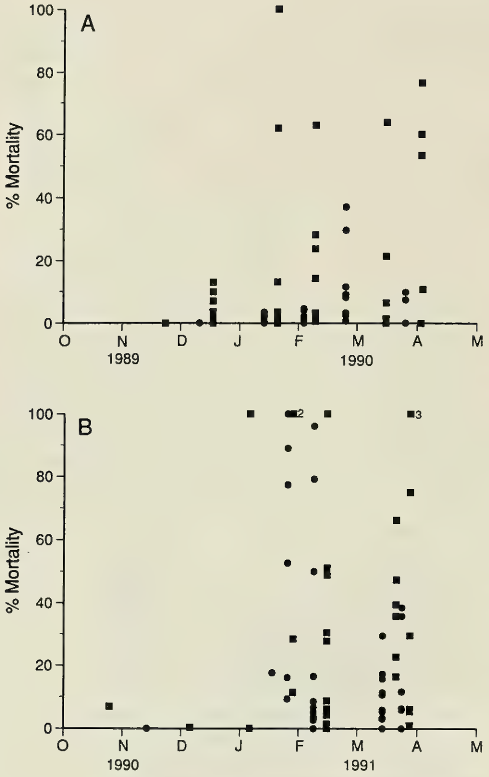

Figure 2. Percent mortality of pentactulae during brooding in *Leptosynapta clarki* at Site 1 (circles) and Site 2 (squares). (A) Mortality in the 1989–1990 reproductive season. (B) Mortality in the 1990–1991 reproductive season. Points for Site 1 are offset by 5 days to avoid overlapping points. In B, numbers adjacent to values of 100% mortality describe the number of observations with that value.

Table II

*Instantaneous mortality rates (*M*, day^{-1}) for pentactulae brooded in female* Leptosynapta clarki *in March and April 1990, 1991*

Date	Mean M	SD	Minimum M	Maximum M	n
Mar. 1990	−0.0015	0.0027	−0.0108	0	18
Apr. 1990	−0.0034	0.0043	−0.0114	0	8
Mar. 1991	−0.0022	0.0025	−0.0094	0	17
Apr. 1991	−0.0018	0.0031	−0.0114	0	14*

Values for t ranged from 93 to 128 days. Data are pooled from Sites 1 and 2. Minimum and maximum M and measures of the highest and lowest values of instantaneous mortality rate respectively. A value of M = 0 reflects no pentactulae loss.

* Three females had no living pentactulae (1, 1, 3 dead pentactulae or rings only = 100% mortality). Values for M could not be calculated as $(N_o/N_t) = 0$, and are omitted from the mean value of M.

SD: 30.7 ± 34.7 pentactulae, range 1–99, $N = 9$) than females with < 100% mortality (mean ± SD: 173.5 ± 172.9 pentactulae, range 1–839, $n = 146$; $t = 7.761$, $P > 0.05$). Two females each contained only a single dead pentactula.

Discussion

The retention of the calcareous ring of dead pentactulae in the ovarian tubules of *Leptosynapta clarki* has provided a novel system for studying mortality in a viviparous marine invertebrate. The persistence of this ring makes it possible to determine how many pentactulae were present at the beginning of the brooding period. Two assumptions have, however, been made in the present study: (1) Mortality is negligible prior to the pentactula stage with a calcareous ring. Any mortality that occurs prior to this stage cannot be differentiated from resorption of unfertilized eggs, so this method may slightly underestimate mortality. However, because pentactulae hatch in the ovarian lumen within 10–14 days of fertilization, the calcareous ring is present for about 93% of the 30-week brooding period (Sewell and Chia, 1994). (2) No dissolution of calcareous rings occurs within the ovarian tubule. No evidence was found for resorption of the calcareous rings during the brooding period; in some dead pentactulae, the connective

Table III

Tests for relationship between maternal features and percent pentactulae mortality

	Drained weight (mg)			No. pentactulae brooded			
Date	F	P	r^2	F	P	r^2	n
Jan. 1990	0.016	0.900	0.001	4.851	0.044	0.244	17
Feb. 1990	0.094	0.764	0.006	2.889	0.109	0.152	18
Mar. 1990	0.0004	0.985	2.3×10^{-5}	0.297	0.593	0.018	18
Apr. 1990	2.140	0.194	0.263	0.761	0.417	0.113	8
Jan. 1991	2.659	0.164	0.347	0.166	0.701	0.032	7
Feb. 1991	1.232	0.276	0.039	9.272**	0.005	0.236	32
Mar. 1991	5.325	0.036	0.262	2.117	0.166	0.124	17
Apr. 1991	0.030	0.865	0.002	2.164	0.162	0.126	17

Ordinary least squares regressions were calculated between percent mortality and (1) female size (drained weight) and (2) total number of pentactulae (No.) brooded. Regressions are based on pentactulae mortality from Sites 1 and 2 combined for each sampling time in 1990 and 1991. Values given are F-statistic (F), probability for F-statistic (P) and r^2 for regression. Statistical significance determined within each set of comparisons using a sequential Bonferroni test (Rice, 1989) and a groupwide Type 1 error of 0.05. Significance of regression: no symbol = NS; ** = $P < 0.01$.

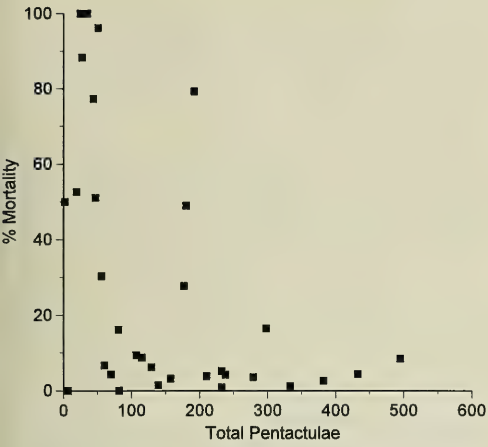

Figure 3. Relationship between total pentactulae number (No.) and percent mortality of brooded pentactulae in female *Leptosynapta clarki* in February 1991.

tissue holding the pieces of the ring was resorbed, but the pieces of the ring always remained in a pile in the tubule. Although some underestimation of mortality may result from these assumptions, the fact that the calcareous rings are retained within a discrete structure (the tubelike ovary) and the pentactulae are released at the same time (Sewell and Chia, 1994) means that there is no error in N_o due to loss of embryos in sampling or release or migration of early-maturing juveniles (Hendler and Franz, 1982; Rumrill, 1982).

Studying embryonic mortality in a brooding species allows direct comparison of features of the mother to reproductive success. Such a comparison is not possible with planktonic mortality rates, which are based on a mixture of offspring from a number of females (see reviews by Chaffee and Strathmann, 1984; Strathmann, 1985; Hines, 1986; Rumrill, 1990). Strathmann and Strathmann (1982) suggested that larger broods from larger adults might develop more slowly or suffer higher mortality during brooding. The data here, however, show weak relationships between embryonic mortality and maternal features such as size or number of pentactulae brooded. Additionally, when considering the entire data set, no maternal feature consistently separated females with extremely high mortality from other females.

Instantaneous mortality for female *L. clarki* with at least one living pentactulae was -0.0021 d^{-1}. This value is similar in magnitude to estimates for other brooding marine invertebrates (-0.0046 to -0.0523 d^{-1}; reviewed in Rumrill, 1990). When making such a comparison, however, we need to consider (1) the assumptions used in determining N_o in previous studies, (2) that mortality rates may not be constant over time (Hendler and Franz, 1982; Chaffee and Strathmann, 1984; present study), and (3) that additional mortality may result from mortality of the mother during the brooding period. Loss of females during brooding may occur due to predation (Emson and Crump, 1976), dislodgement (Menge, 1974), or osmotic stress during rainfall (in *L. clarki,* pers. obs.).

The data from this viviparous species suggest that pentactulae mortality in female *L. clarki* is variable, and can be extremely high in some females. The reasons for death of pentactulae within the ovary are at present unknown. Pentactulae might die because of insufficient supplies of food or oxygen, genetic abnormalities, or the buildup of waste products within the ovary. Once dead, however, they may be a source of nutrition for the remaining pentactulae. Initially, nutrition is supplied from the breakdown of unfertilized eggs (Everingham, 1961; McEuen, 1986; Sewell and Chia, 1994). Later in the brooding period no discrete droplets are visible in the tubules and the pentactulae feed on the fluid itself (Everingham, 1961; Sewell and Chia, 1994). Resorption of the dead pentactulae may be important in providing additional organic material to the ovarian fluid.

The death of embryos in *L. clarki* during the brooding period has different consequences from mortality during other forms of brooding in that the resorbed material is available to living siblings in the ovary, to the mother, or to both. Internal brooding (*e.g.,* in the coelom) and viviparity are at the extreme end of a nutritional continuum. At the other end of the continuum are pelagically developing larvae (both planktotrophic and lecithotrophic) which gain little or no nutrition from the death of siblings. In an intermediate position on the continuum are external brooders or species in which the brood structure is open to seawater (*e.g.,* bursal brooding in ophiuroids, brood pouches). In the intermediate case, some material might be available to siblings or the mother, but resorbing material is likely to be removed from the area of brooding due to dissolution in seawater.

Mortality of embryos in internal brooding and viviparous species should be considered in life-history models as a special type of mortality. In these cases, embryo death returns the initial maternal investment in that egg or embryo to the remaining siblings or to the mother. Embryo mortality or sibling cannibalism in viviparous echinoderms (*e.g., Patiriella vivipara, P. parvivipara;* Byrne, 1996) might be important for survival of the remaining embryos and perhaps allow growth to a larger size at release. In *L. clarki* the smallest broods have the largest median pentactula size (Sewell, unpub. data). If there is an advantage to being released at a larger size (Smiley *et al.,* 1991), then additional nutrition provided by resorption of dead embryos, or from the mother to a smaller number of pentactulae, might have fitness consequences for the juveniles after release.

Because most species probably experience highest mortality during their early developmental period, Vance (1973) concluded that "those forces affecting larval mortality probably play a very important role in the evolution of the general pattern of reproduction" (p. 339). Thus to understand the selective advantages of each life-history strategy, we must continue to collect information on embryo mortality in species with a wide range of reproductive patterns. The method described here, based on retention of a hard embryonic structure, is potentially applicable to measuring mortality in internal brooders (*e.g.*, gill-brooding bivalves). Qualitative descriptions of embryo mortality have been reported in two other internally brooding echinoderms. A sphere observed among the intraovarian embryos of the crinoid *Comatilia iridometriformis* might represent an embryo undergoing resorption (Messing, 1984). Degenerating embryos observed fused to the internal surface of adult *Ophionotus hexactis* (Morison, 1979) may have been too large to exit the bursae (Turner and Dearborn, 1979), or alternatively may have died earlier in development. Careful dissections in these and other brooding species may provide further estimates of mortality during brooding.

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Vertical Transmission of Chemoautotrophic Symbionts in the Bivalve *Solemya velum* (Bivalvia: Protobranchia)

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Abstract. Adults of the bivalve species *Solemya velum* live in symbiosis with chemoautotrophic bacteria in specialized gill bacteriocytes. The bacteria play an essential nutritional role in the mature association, fixing CO_2 via the Calvin cycle with energy obtained through the oxidation of reduced sulfur compounds. To understand how the continuity of this partnership is maintained between host generations, we investigated the mode of symbiont transfer in *S. velum.* A diagnostic assay using the polymerase chain reaction and primers specific for the *S. velum* symbiont ribulose-1,5-bisphosphate carboxylase (RubisCO) gene consistently detected bacterial sequence in female gonad tissue, suggesting the presence of symbiont cells in host ovaries and a vertical mode of symbiont transmission from mother to offspring. Furthermore, intracellular bacteria were present in the developing gills of juveniles that had not yet hatched from the gelatinous capsule in which larval development occurs (11 days after fertilization). By 64 days postfertilization, the typical adult gill ultrastructure of alternating bacteriocytes and symbiont-free intercalary cells was apparent. Knowledge about the mode of symbiont transfer in *S. velum* allows further study into the dynamics of host-symbiont interactions in chemoautotrophic associations.

Introduction

Chemoautotrophic symbioses are nutritionally based mutualistic associations between sulfur-oxidizing chemoautotrophic bacteria and marine invertebrate hosts. As autotrophs, the symbionts synthesize organic matter from CO_2 and are the primary source of nutrition for the whole organism. In turn, the animal host provides its symbionts an expanded habitat in which they have access to the substrates of chemoautotrophy (O_2, CO_2, and reduced inorganic compounds). Together, these partners create animals with novel metabolic capabilities that allow them to colonize relatively hostile environments. Deep-sea hydrothermal vent communities are dominated by chemoautotrophic symbioses and are perhaps the most dramatic examples of the success of these types of organisms, although similar associations are found in a number of invertebrate host phyla occupying a range of reducing habitats (Cavanaugh, 1994; Fisher, 1990).

All symbiotic associations must meet the challenge of maintaining the animal–bacteria partnership over successive host generations (Smith and Douglas, 1987). Symbiont transfer may be achieved through external transfer, in which offspring are born symbiont-free and are infected from the environment; through horizontal transfer, in which offspring are born symbiont-free and are infected by other members of the parent population; or through vertical transfer, in which gametes (most commonly oocytes) carry symbionts either intra- or extracellularly to the succeeding generation (Buchner, 1965). Although transmission mechanisms have been studied in only a few chemoautotrophic species, they appear to be as varied as the host species themselves. External transfer has been inferred for hydrothermal vent tube-worms, for instance, because symbionts have never been found in host reproductive tissues (Cary *et al.*, 1993; Cavanaugh *et al.*, 1981). In contrast, a variety of vertical transmission mechanisms have been inferred for oligochaete and bi-

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valve hosts. Females of the oligochaete species *Inanidrilus (Phallodrilus) leukodermatus* and *I. planus* apparently infect their offspring by smearing symbionts onto the eggs as they pass across symbiont-containing genital pads into the sediments (Giere *et al.,* 1991). Female vesicomyid clams and the gutless bivalve *S. reidi,* in contrast, appear to produce eggs with intracellular symbionts. Evidence in the vesicomyids is based on inclusions resembling gram-negative bacteria that were observed in the oocytes of *Calyptogena soyoae* with transmission electron microscopy (Endow and Ohta, 1990). For *S. reidi,* polymerase chain reaction (PCR) experiments were used to establish the presence of symbionts in host ovaries, eggs, and larvae (Cary, 1994). This, together with the subsequent *in situ* identification of symbionts in *S. reidi* test cells, was interpreted as consistent with the transovarial transmission hypothesis proposed by Gustafson and Reid (1988b; Cary, 1994).

The present study addresses the question of symbiont transfer in the chemoautotrophic bivalve *Solemya velum. S. velum* lives in shallow-water sediments from Nova Scotia to northern Florida (Vokes, 1955) and, because of its relative accessibility, has been extensively studied as a model chemoautotrophic symbiosis. The recent description of the host life cycle (Gustafson and Lutz, 1992), however, has made it clear that *S. velum* is different from other hosts in which reproduction and symbiont transfer have been studied. In sharp contrast to most marine bivalves, including the congener *S. reidi, S. velum* does not have a planktonic phase. Following fertilization in the water column, each embryo is enclosed in a negatively buoyant, gelatinous capsule that rests on the sediment surface. Larval development proceeds within this capsule through embryonic, pericalymma larval, and postmetamorphic juvenile stages. Juveniles hatch as fully formed clams capable of burrowing directly into the mud. At hatching, at least three gill filaments have been formed, but it is not known whether they contain bacterial symbionts (Gustafson and Lutz, 1992).

In adult *S. velum,* symbionts populate host gill cells known as bacteriocytes, where they are contained within membrane-bounded vacuoles and reach densities of about 10^9 bacteria per gram wet weight of gill (Cavanaugh, 1983). Based on 16S rRNA studies, the entire symbiont population is composed of a single bacterial species unique to the *S. velum* host (Eisen *et al.,* 1992). According to feeding (Krueger *et al.,* 1992) and stable isotope (Conway *et al.,* 1989) studies, the symbionts are the primary source of nutrition to the mature symbiosis.

In light of the obligate nature of the symbiosis with respect to nutrition and the direct developmental pattern of the host, the hypothesis of vertical symbiont transmission was proposed for *S. velum.* Several experimental approaches were used in testing this hypothesis, which ultimately lead to the development of an assay using PCR for detecting symbionts in host tissues. Studies using microscopy and *in situ* hybridization methods gave ambiguous results due to the difficulty of detecting and identifying symbionts that are apparently present in small numbers or are metabolically quiescent in host reproductive tissues. In contrast, use of PCR, in combination with primers identical to an *S. velum* symbiont gene, resulted in an extremely sensitive and specific assay for screening *S. velum* gonads. Larval gills were also examined with electron microscopy to document the symbiosis in the early life stages of a new host generation.

Materials and Methods

Specimens

Adult *Solemya velum* greater than 0.7 cm in length were collected from eelgrass beds near Woods Hole, Massachusetts, between September 1992 and October 1993. These collections provided a thorough sampling of the population throughout the seasonal changes typical of the habitat and the potential seasonal reproductive pattern of the host. Collection months were September and December 1992, and January, April, May, June, July, and October 1993. Juvenile specimens (11 and 64 days postfertilization) were obtained from cultures of a laboratory-spawned population as described in Gustafson and Lutz (1992). The 11-day-old specimens had not yet hatched from the gelatinous capsule in which embryonic development occurs, while at 64 days, the clams had been free of the capsule for about 7 weeks. Immediately following collection, all specimens were processed either for microscopic or molecular studies (see below).

Cultures of the free-living, sulfur-oxidizing, chemoautotrophic bacterium *Thiomicrospira* sp. str. L12 and DNA from the grass *Secale cereale* used in testing PCR primer specificity (see below) were generously provided by C. Wirsen and H. Jannasch (Woods Hole Oceanographic Institution), and T. Kellogg (Harvard University), respectively.

Nucleic acid extraction

In each of the collection months, tissues of individual *S. velum* were dissected for nucleic acid extraction. To reduce the possibility of contamination during dissection, UV-sterilized equipment was used to sequentially remove foot (symbiont-free), gonad, and gill (symbiont-containing) tissues from individual specimens. The tissues were frozen in liquid nitrogen and stored frozen at −80°C. Nucleic acids were extracted from these tissues by using a guanidinium isothiocyanate (GITC) isolation method modified from Ausubel *et al.* (1990). Tissues were homogenized in 5 *M* GITC extraction buffer (1:10, wt:vol) followed by several phenol:chloroform (1:1) extractions to remove denatured proteins and other cellular debris. Contaminating phenol was removed with several more chloroform extractions. DNA was recovered by ethanol precipitation, and final concentrations were determined spectrophotometrically. Extraction products were visualized with agarose gel electrophoresis and ethidium bromide staining to confirm the presence of high molecular weight DNA. All DNA samples were stored frozen at −20°C.

PCR detection of symbiont genes

To determine whether symbionts are transmitted vertically in *S. velum,* nucleic acids extracted from reproductive tissues of adult clams were screened for the presence of symbiont genes. For screening, PCR (Mullis and Faloona, 1987) was used in combination with primers specific to the symbiont ribulose-1,5-bisphosphate carboxylase (RubisCO) gene. RubisCO is specific to autotrophs that use the Calvin cycle (Tabita, 1988) and is the key enzyme for carbon fixation in the symbionts of *S. velum* (Cavanaugh *et al.,* 1988). Furthermore, the *S. velum* symbiont RubisCO gene has recently been sequenced (unpub. data), so that primers identical to the gene could be designed. Because it involves amplification of the target sequence, PCR allows for the highest degree of sensitivity possible in detecting bacterial genes within host gonads, and the use of symbiont-specific primers maximizes specificity of the reaction. The advantages of a PCR-assisted screening approach, therefore, include extreme sensitivity and specificity that cannot be achieved with more traditional methods such as microscopy.

PCR primers (18 mers) identical to the *S. velum* symbiont RubisCO sequence (unpub. data) and with a minimum of five mismatches to other available RubisCO sequences were designed to amplify the 430-1058 region (629 bp) of the *S. velum* symbiont RubisCO gene (numbering based on *Spinacia oleracea* (Schneider *et al.,* 1990)): 430F ($^{5'}$TACGTAATGACCTGTAAT$^{3'}$; sense primer), and 1058R ($^{5'}$AATGAATCACGCATGATA$^{3'}$; antisense primer). Conditions for PCR were optimized using nucleic acids isolated from adult *S. velum* gills, where the symbionts reside in large numbers (Cavanaugh, 1983; Cavanaugh *et al.,* 1988). In the optimized protocol, each PCR reaction mixture contained 0.1 μM of each primer, 2.5 μM dNTPs, 2 mM MgCl$_2$, 150 ng DNA, and 0.125 μl Taq polymerase (Promega) in a total volume of 25 μl. The thermocycler (Ericomp) parameters were 94°C (2 min) + (94°C (1 min), 52°C (1 min), 72°C (1 min)) for 35 cycles. The detection limit of the assay was determined with serially diluted gill DNA samples, which contained both eukaryotic (host) and bacterial (symbiont) genes. Consistent detection of the symbiont RubisCO gene was not possible below a total DNA starting concentration of 2.5 ng.

The PCR assay was run on DNA extracted from the gonads of three to seven female *S. velum* and one male from each sampling month except September and December 1992, when no adult males were processed. DNA from *S. velum* gill and foot tissue served as positive and negative assay controls, respectively. Duplicate reactions were performed on each sample. Results were determined by visualizing PCR products with agarose gel electrophoresis and ethidium bromide staining; a sample was scored as a positive (+) PCR if a product of the predicted size (629 bp) was present in at least one of the replicate samples.

Specificity of the 430F/1058R primer set was tested on a variety of RubisCO-containing samples: gills of *S. velum* and *S. reidi* (a congener also living in symbiosis with chemoautotrophic bacteria (Felbeck, 1983)); the free-living chemoautotrophic bacterium *Thiomicrospira* sp. str. L12; and the grass *Secale cerale.* The presence of RubisCO in these samples was verified by using PCR primers 37F and 1406R, designed to amplify Form I RubisCO corresponding to the 37-1406 (1370 bp) region of *S. oleracea* from a wide range of autotrophs (unpub. data). The product amplified from an *S. velum* gonad sample with the 430F/1058R primer set was partially sequenced (≈240 bp) to confirm its identity as the *S. velum* symbiont RubisCO. After column purification (Cetus Corp.) of the PCR reaction product, the Sequenase V.2 (USB) protocol for double-stranded sequencing was followed.

Microscopy

To verify that symbiotic bacteria are present in the early life stages of *S. velum* and to document the development of the symbiosis in a new generation of hosts, juveniles collected from laboratory-spawned cultures 11 and 64 days after fertilization (Gustafson and Lutz, 1992) were examined. The specimens were fixed in 2% glutaraldehyde in 0.2 M sodium-phosphate buffer and 0.14 M NaCl (pH 7.3), and postfixed in 2% OsO$_4$ in 1.25% NaCO$_3$. The 11-day-old samples were mechanically removed from their capsules according to the method described by Gustafson and Lutz (1992). All specimens were embedded in Spurr's resin (Spurr, 1969) in accordance with established protocols (Cavanaugh, 1983; Cavanaugh *et al.,* 1988). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7000 transmission electron microscope.

Results

Primer specificity

The PCR primers designed to amplify a 629 bp product from the *Solemya velum* symbiont genome were shown to be specific for the symbiont RubisCO gene. Although the general Form I RubisCO primers 37F/1406R amplified a 1370 bp product from a variety of RubisCO-containing samples, the specific primers 430F/1058R yielded product only from *S. velum* sample (Fig. 1). This product was confirmed through sequence comparison to be identical to the *S. velum* symbiont RubisCO sequence (data not shown). It was therefore concluded that the newly developed PCR assay is a useful diagnostic test for the presence of *S. velum* symbionts in host tissues.

Symbionts in host ovaries

Nucleic acids extracted from *S. velum* samples collected throughout the year from September 1992 to October 1993 were screened using PCR with the symbiont-specific RubisCO primers 430F/1058R to test the hypothesis of vertical transmission in *S. velum.* The results of the PCR assay on July 1993 samples are typical of all other months tested (Fig. 2). PCR products of the expected size of 629 bp were amplified from the female gonad and gill (run as positive control) samples; no products were detectable when DNA from the foot (negative control) and male gonad was used as template. Symbiont sequence was amplified from every female clam gonad tested (Table I), with the exception of single specimens in May and June (see Discussion). Symbionts were never detected in male gonad or foot samples, indicating that the positive results are probably not due to surface contamination. Detection

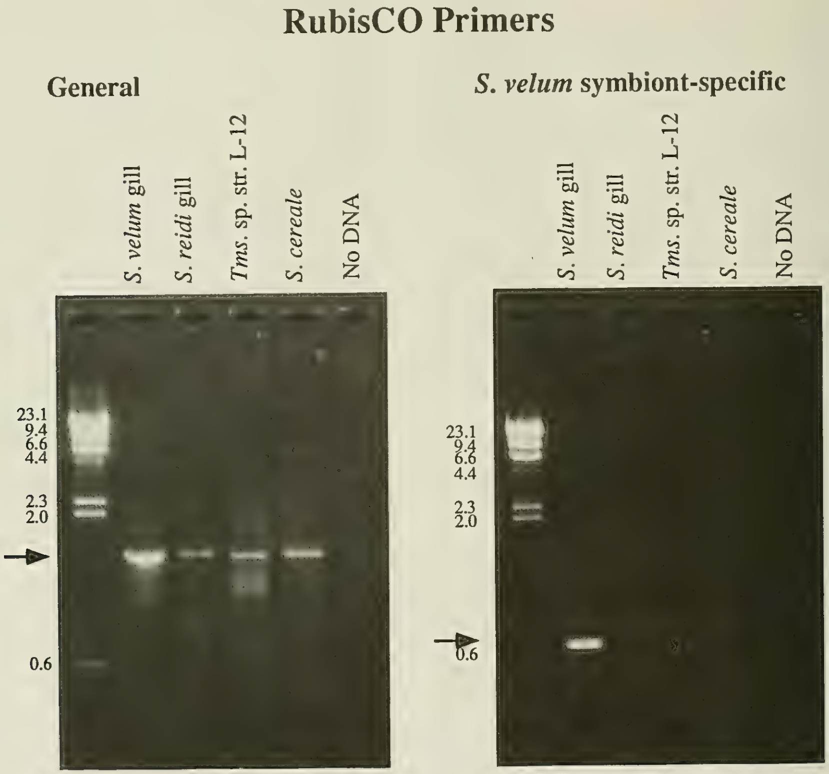

Figure 1. Agarose gel (1%) showing the specificity of PCR primers for the *Solemya velum* symbiont RubisCO gene. DNA samples from symbiont-containing *S. velum* gill tissue (lane 2), symbiont-containing *S. reidi* gill tissue (lane 3), *Thiomicrospira* sp. str. L12 (lane 4), *S. cereale* (lane 5), and negative controls with no DNA (lane 6) were submitted to PCR amplification with (A) the general RubisCO primers 37F/1406R (1370 bp product; arrow) or (B) the *S. velum* symbiont-specific RubisCO primers 430F/1058R (629 bp product: arrow). Lane 1 contains the molecular weight standard Lambda HinDIII with band sizes (kb) indicated to the left.

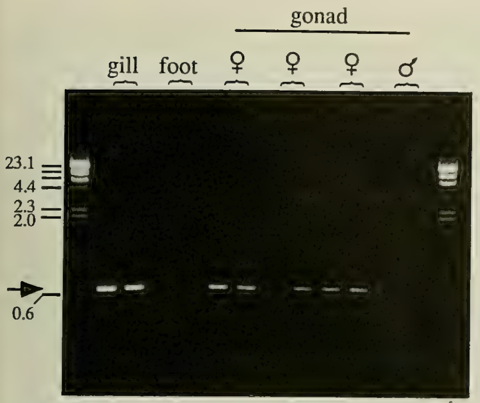

Figure 2. Agarose gel (1%) showing PCR amplifications of a 629 bp segment (arrow) of the symbiont RubisCO gene from host gonads collected in July 1993 with the *Solemya velum* symbiont-specific 430F/1058R primers. Gonad samples from 3 females (lanes 5–10) and 1 male (lanes 11–12) were each run in duplicate and considered positive when product of the correct size was visualized in at least one replicate reaction per individual sample. Positive and negative controls were samples of *S. velum* gill (lanes 1–2) and foot (Lanes 3–4), respectively. Lambda HinDIII molecular weight marker shown in first and last lanes.

of a symbiont gene with such a specific and sensitive assay suggests that symbionts are present in the ovaries of *S. velum* throughout the year.

Juvenile host gills

Bacteria are present in gills of *S. velum* that are as young as 11 days postfertilization and which are still developing inside the egg capsule. The gill buds of these young clams are just beginning to form filaments, and subcellular inclusions resembling prokaryotic cells can be identified inside the unciliated cells located near the base of the filaments (Fig. 3A). As is typical of adult *S. velum* symbionts, the bacteria in these larvae have a gram-negative bacterial cell envelope and are surrounded by a third, outer membrane presumed to be derived from the host. Many of the bacteria appear to be dividing.

In 64-day-old clams, the gills are composed of numerous filaments and resemble adult *S. velum* gills with respect to their ultrastructure. Bacteriocytes (cells containing many bacteria) and intercalary cells (symbiont-free) are readily identifiable in the region proximal to the ciliated edge of each filament, which is also symbiont-free (Fig. 3B). This bacteriocyte region of the filament is characteristically covered with microvilli. Like the bacteria in the gills of the adult, those in the gills of 64-day-old juveniles have gram-negative cell envelopes, are contained within individual membrane-bounded vacuoles, and are located in the apical region of the bacteriocytes.

Discussion

The use of PCR in combination with primers identical to the *Solemya velum* symbiont RubisCO gene made it possible to establish the presence or absence of symbionts in host tissues and thereby test for vertical transmission of symbionts in this clam. Initially, *S. velum* oocytes were examined with transmission electron microscopy, but it was impossible to identify any membrane-bounded inclusions as bacterial symbionts on the basis of ultrastructure alone. Pinocytotic vesicles and yolk granules are common in molluscan eggs and can look remarkably similar to gram-negative bacteria in a host vacuole at the ultrastructural level (*cf.*, figure 16, deJong-Brink *et al.*, 1984). By designing an assay to recognize *S. velum* symbionts on the basis of molecular characteristics (Fig. 1), the necessary sensitivity and specificity were achieved. PCR methods similar to ours have been used for diagnostic purposes in the agricultural (Sawada *et al.*, 1995) and environmental (Steffan and Atlas, 1991; Voytek and Ward, 1995) sciences, in medicine (Webb *et al.*, 1990), and in other studies of symbiosis (Cary *et al.*, 1993; Cary, 1994; Rouhbakhsh *et al.*, 1994).

The *S. velum* symbiont RubisCO gene was consistently amplified from DNA extracted from female clam ovarian tissue collected throughout the year, but no product was ever amplified with the same assay on samples of *S. velum* foot or male gonad (Fig. 2, Table I). In two female gonad samples, no amplification product was observed. Although

Table I

Detection of bacterial symbionts in DNA extracted from Solemya velum *gonad tissue collected near Woods Hole, Massachusetts, between September 1992 and October 1993 using PCR with primers specific for the* S. velum *symbiont RubisCO gene*

Collection		No. females tested	No. females +PCR	No. males tested	No. males +PCR
Year	Month				
1992	September	3	3	—	—
	December	3	3	—	—
1993	January	3	3	1	0
	April	3	3	1	0
	May	7	6	1	0
	June	4	3	1	0
	July	3	3	1	0
	October	4	4	1	0

Positive PCR (+PCR) results were determined by visualizing PCR products of the correct size (629 bp) in at least one replicate sample with agarose gel electrophoresis and ethidium bromide staining. For each PCR assay, gill and foot were tested as positive and negative controls, respectively.

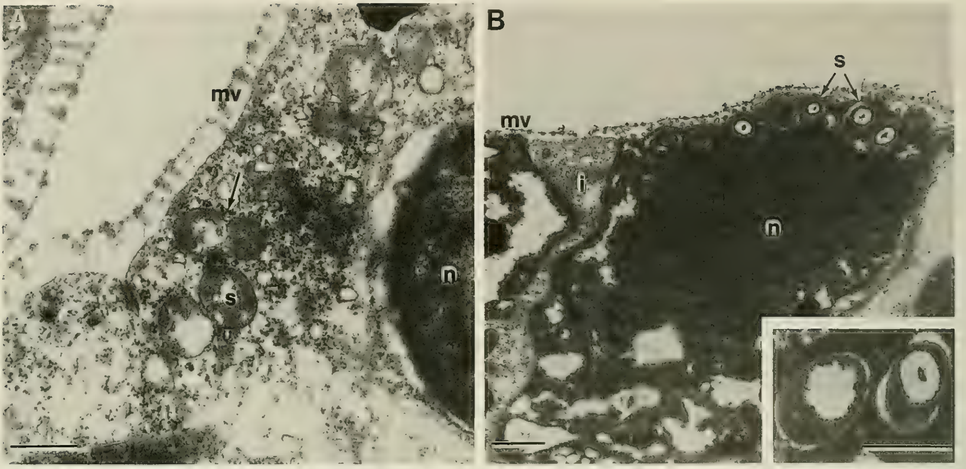

Figure 3. Transmission electron micrographs showing bacteria inside juvenile *Solemya velum* gills cells. (A) Section of 11-day-old gill with inclusions resembling gram-negative bacteria in nonciliated cells at the base of developing filaments. Many of these bacteria appear to be dividing (arrow). Scale bar = 0.5 μm. (B) Longitudinal section through a single filament of a 64-day-old individual showing alternating symbiont-containing bacteriocytes and symbiont-free, goblet-shaped intercalary cells along the filament proximal to the ciliated edge. The bacteria are concentrated near the outer edge of the host cell, have gram-negative cell walls, and are contained within individual membrane-bounded vacuoles (inset). Scale bars = 1 μm. i = intercalary cell, n = bacteriocyte nucleus, s = symbiont, mv = microvilli.

this result could be caused by technical inconsistencies in the assay, an alternative explanation is that these gonad samples had too few bacteria to yield a PCR product. This could be the case if, for example, the clams had recently spawned. Overall, the data suggest that female *S. velum* maintain a continually maturing population of oocytes with a seed population of symbionts. This conclusion is consistent with the observed intimacy of the adult symbiosis and the hypothesis that *S. velum* symbionts are transmitted vertically from host generation to generation. Cary (1994) used a similar approach of screening host tissues with PCR and symbiont-specific primers in his study of symbiont transmission in the congener species *S. reidi*. Transovarial symbiont transfer was also hypothesized for *S. reidi* on the basis of its nutritional dependence on the chemoautotrophic symbionts in its gills (Fisher, 1990)—adult *S. reidi* completely lack both mouth and gut! (Reid, 1980). Positive PCR amplification of symbiont genes from DNA extracted from host gills, ovaries, eggs, and larvae indicated that this was indeed the case (Cary, 1994). Unlike *S. reidi,* however, *S. velum* could not be reliably spawned in the laboratory, so our PCR screening of *S. velum* tissues was limited to whole clam gonads. Therefore, the symbiont sequence we detect with PCR in the female gonad samples may be due to bacterial symbionts inside or outside of the oocytes. *S. velum* could produce eggs carrying intracellular bacteria, as was shown for *S. reidi* (Cary, 1994), or deposit its symbionts on the outsides of the eggs during spawning, as in symbiotic oligochaetes (Giere *et al.,* 1991). The fact that no symbiont genes were amplified from either *S. velum* foot tissue or testes implies, however, that symbionts are transmitted across host generations *via* the female germ line.

Most marine invertebrates have a free-living larval stage, and it is during this phase that many symbiotic species are infected by nonvertical mechanisms of symbiont transfer (Trench, 1979). *S. velum* larvae, however, develop inside of a gelatinous egg capsule so that contact with the external environment during early development is relatively restricted (Gustafson and Lutz, 1992). Larval egg capsules are produced by many bivalve species (*e.g.,* Alatalo *et al.,* 1984, and references therein; Gibbs, 1984; Gustafson and Reid, 1988a), and are thought to function primarily as protection devices during development and dispersal (Pechenik, 1979). Exchange with the external environment is generally considered to be limited to water and salts (Hawkins and Hutchinson, 1988). Furthermore, scanning electron microscopy of the surface of the *S. velum* egg capsule did not show any pores through which potential symbionts could enter (unpub. data). The presence of bacteria in host ovaries (Table I) and in the gill cells of *S. velum* still confined to the egg capsule (Fig. 3A), therefore, indicates that the symbiosis is established very early in the life cycle of the host animal, and is consistent with the hypothesis of vertical symbiont transmission in this species.

Within the gills of the adult host, *S. velum* symbionts are metabolically active, oxidizing sulfide (Chen *et al.*, 1987) and fixing CO_2 (Cavanaugh, 1983; Cavanaugh *et al.*, 1988) into organic carbon for the whole organism. The metabolic state of the symbionts contained within gonadal tissue is unknown, however. In the present study, many attempts were made to detect *S. velum* symbionts in host ovaries by using *in situ* hybridization with a symbiont 16S rRNA-directed oligonucleotide probe (Distel and Cavanaugh, 1994; Eisen *et al.*, 1992). Whereas *in situ* labeling of host gills—where bacteria are numerous and metabolically active—was consistently strong, labeling in gonad tissue was extremely weak to absent (unpub. data). Ribosome number is directly correlated with growth in bacteria and, accordingly, so is *in situ* labeling intensity when rRNA probes are used (Amann *et al.*, 1995). The failure of this method to detect symbionts in *S. velum* gonads may be due to low numbers of bacteria, to the metabolic quiescence of symbionts in these tissues, or to a combination of both factors. This scenario is consistent with the fact that *S. velum* spawns large ($\approx$400 μm diameter), yolky eggs, and the larvae develop lecithotrophically while inside the egg capsule (Gustafson and Lutz, 1992).

It is now apparent that bacterial symbionts are integrated into the entire life cycle of *S. velum* given their presence in reproductive tissue (ovaries [Fig. 2, Table I]), larvae (Fig. 3A), juveniles (Fig. 3B), and adults (Cavanaugh, 1983). Our observations are consistent with the hypothesis that female hosts transmit the bacterial symbionts to new generations, and that bacteria derived from this seed population colonize the gills as filaments are formed in juvenile clams. The functional role of the symbionts in young *S. velum* hosts remains unknown, however. The symbionts are probably not metabolically active before and just after spawning, but they colonize the gill buds and appear to be actively growing while still within the larval egg capsule. Dividing bacteria were relatively common in the gills of 11-day-old clams (Fig. 3A)—more so than in the adult—suggesting rapid expansion of the symbiont population during the early development of the host. By 2 months postfertilization, the symbiosis is well established in the new host generation and closely resembles the adult association both morphologically and ultrastructurally (Fig. 3B). The question arises as to whether these juvenile hosts are also already nutritionally dependent on their symbionts, or whether they assimilate exogenous material. Adult *S. velum* possess a fully functional digestive system, but they feed little on suspended particles and depend almost entirely on their symbionts for nutrition (Krueger *et al.*, 1992). Efforts are currently under way to characterize the nutritional significance of chemoautotrophic symbionts through ontogeny in this model host.

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Visual Pigment Types and Quantum-catch Ratios: Implications From Three Marine Teleosts

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Abstract. Experimental data on photoreceptor cells and visual pigments are the basis for model calculations performed to assess photoreceptor quantum catches under disparate irradiance conditions. Three unrelated species of fish—black sea bass (*Centropristis striata*), sea raven (*Hemitripterus americanus*), and adult winter flounder (*Pseudopleuronectes americanus*)—are considered. In each case, receptor type quantum catches are compared for various water types and depths. By associating the habit and habitat of an organism with the physical properties of its photoreceptors, quantum-catch ratios are found as possible criteria in the selection of pigment peaks (λ_{max}). In addition to integrated ("total") quantum catches by the receptor types, rates of quantum catches are determined as a function of wavelength. The latter functions are replotted as pairwise difference spectra. These, in turn, are used to assess the ability of receptor types to participate in wavelength discrimination.

Introduction

Vertebrate vision is mediated by retinal photoreceptor cells equipped with specific sensory pigments. The primary role of these pigments is to detect light—a form of electromagnetic radiation that carries spatial, temporal, and other types of information about the environment. When the information to be extracted from the incident photons concerns their wavelength distribution, we are in the realm of color vision. By definition, color vision refers to the ability of an eye to discriminate between light stimuli of varied wavelength composition independent of intensity. As discussed below, wavelength discrimination requires the action of at least two receptor types with spectrally disparate visual pigments. Primarily on the basis of microspectrophotometric determinations, we know that a given retina may contain one to five such pigments (*e.g.*, Robinson *et al.*, 1993). Moreover, there are a hundred or more spectral types of pigment with distinct peaks (λ_{max}) to chose from. Two questions thus arise: What are the reasons for choosing a certain number of pigment types? How can we rationalize the existing set of pigments with specific λ_{max} values to be found in a given organism? We do not have the final answers to these questions. The present study was undertaken to chart a spectroscopist's approach to the problem.

Retinal rod and cone cells are known to be quantum detectors: they respond to light by giving an output signal proportional to the number of photons absorbed. But photoreceptors cannot sense the wavelength of light directly, because they do not utilize the energy of the absorbed photons. Instead, light serves merely to trigger the photoresponse, the magnitude of which is the consequence of spectral absorption by the sensory pigment. The output signal of each photoreceptor is a product of two wavelength-dependent functions: the spectral characteristic of the receptor pigment and the spectral content of the light stimulus. Even when the characteristic function of the pigment is fixed, each response derives from the integral of all absorbed photons over all wavelengths. Because visual pigments are wide-band absorbers, photoreceptor outputs have no unique interpretations for the spectral content of the stimulus. For this reason, two or more receptor types with disparate pigment characteristics are needed. By sensing the same stimulus, these give disparate responses and analysis of the differences in response provides information about spectral distribution. On the basis

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of this knowledge, we may engineer a wavelength discriminator that uses two light detectors with overlapping but disparate spectral sensitivities and combines their output signals in a subtractive manner. The output of such a discriminator would yield zero for the neutral point, at which both detectors have equal responsiveness; a positive signal in the spectral region where the first detector's output is greater than that of the second; and a negative signal over the band where the second detector has the larger response. Because horizontal cells (S-potentials) conform to this strategy, we may regard photoreceptor types, each containing a distinct visual pigment, as so many spectral (color) channels that make wavelength discrimination possible.

In a previous study we investigated the retina of the black sea bass for its photoreceptors, the pigments of which we attempted to relate to the habit and habitat of the species (Singarajah and Hárosi, 1992). We characterized its visual pigments by microspectrophotometry. We also made use of published data on daylight spectral irradiance and tables that give transmittance values for natural bodies of water classified into several water types. To assess the interaction between sensory pigment and ambient light, we calculated total photoreceptor quantum catches by integrating the receptor absorptance over the available spectral irradiance spectrum of each habitat. As reported, we found indications that total quantum-catch ratios are of selective value in acquiring pigment types. We suggested that the λ_{max} of a visual pigment in a multipigmented eye may be chosen on the basis of balanced quantum catches to prevail in the receptor types over certain photic habitats of the species.

In this study the former calculations are extended to two other unrelated marine teleosts. Working with model spectra of visual pigments, the generation of which are described, quantum catches by pigment types are assessed. In addition to the calculations of total quantum catches, quantum-catch difference spectra are introduced and their uses are explored from the perspective of wavelength discrimination.

Materials and Methods

Photoreceptor visual pigment data were obtained from both previously published and unpublished determinations. Data for the black sea bass (*Centropristis striata*) were taken from Singarajah and Hárosi (1992). These include the best estimates for λ_{max} of the three pigments and the peak axial absorbance (optical density) of the receptor types (Table I). The data for the sea raven (*Hemitripterus americanus*) were derived from two reports (Collins and MacNichol, 1984; Hárosi, 1982) as well as unpublished results. These were the estimated λ_{max} of the four pigments, the average transverse specific density of the outer segments at $S_{\perp} = 0.016\ \mu m$, and the outer segment length of each receptor type: 8 μm for single cones, 15 μm for each member of double cones, and 20 μm for rods. With these values, the peak axial absorbances were 0.13 for single cones (B), 0.24 for double cones (G and Y), and 0.32 for rods (R). Data for the winter flounder (*Pseudopleuronectes americanus*) were also derived from two sources (Evans *et al.*, 1993; Levine and MacNichol, 1979). Adult specimens of this species have four visual pigments, the λ_{max} of which were estimated from the publications. Video records of isolated photoreceptors provided information on outer segment dimensions. The lengths were found at 6 μm for single cones, 7 μm for each member of double cones, and 15 μm for rods. With an assumed value of 2 for the dichroic ratio, the transverse specific densities were determined from unpolarized absorbance spectra, from which the peak axial absorbances could be calculated. The latter were 0.06 for single cones (B), 0.1 for double cones (G and Y), and 0.25 for rods (R). The primary data serving as the basis for all calculations are summarized in Table I.

Table I

Summary of main parameters used in the calculations: λ_{max} and peak axial optical density of receptor types

	Cone				
Species	Blue B	Green G	Yellow Y	Rod R	Ref.
Black sea bass (*Centropristis striata*)					
λ_{max} [nm]	463	527	—	498	1
Peak axial OD	0.12	0.37	—	0.32	
Sea raven (*Hemitripterus americanus*)					
λ_{max} [nm]	463	522	565	505	2, 3
Peak axial OD	0.13	0.24	0.24	0.32	
Winter flounder (*Pseudopleuronectes americanus*)					
λ_{max} [nm]	457	531	547	506	4, 5
Peak axial OD	0.06	0.1	0.1	0.25	

References
1. Singarajah and Hárosi (1992).
2. Collins and MacNichol (1984).
3. Hárosi (1982).
4. Levine and MacNichol (1979).
5. Evans, Hárosi, and Fernald (1993).

Generation of model spectra for visual pigment α- and β-bands

Experimental spectra are not always available either with sufficiently faithful shape or in wide enough spectral band for the determination of "total" quantum catch. For these reasons I devised a procedure for the simulation of absorbance spectra not only for the α but also extending into the β-band region. The basic provisions for the procedure are that the type of chromophore and the α-band λ_{max} are known. For pigments based on vitamin A_1 (retinal), I assumed that the spectrum obtained from purified bovine rhodopsin by Shichi *et al.* (1969) could serve as a template. I also assumed the validity of the MacNichol-Mansfield relationship that calls for the constancy of α-band shape in the domain of relative absorbance *vs.* relative frequency (MacNichol, 1986).

If a pigment's relative absorbance spectrum (normalized to its α-band peak) *vs.* wavelength (λ) is replotted on a frequency (f) or wavenumber (ν) scale, the original and transformed curves *at equal ordinates* would have abscissae reciprocally related to λ—*i.e.*, $f = c/\lambda$ or $\nu = 1/\lambda$—where c is the speed of light in vacuum. (Because the speed of light has no particular role to play in these considerations and for reason of spectroscopists' tradition, I prefer to work with λ and ν.) The usual definition of half-band width (HBW) is the wavenumber difference corresponding to half-maximum ordinates on either side of the α-band peak, $(\nu_1 - \nu_2)$, which equals numerically $(1/\lambda_1 - 1/\lambda_2)$. If a wavenumber spectrum were now to be replotted on a scale normalized to its peak, ν_{max}, the HBW would become $(\nu_1 - \nu_2)/\nu_{max}$. Moreover, if the α-bands of the various visual pigment spectra were invariant in the domain of relative absorbance *vs.* relative frequency (as suggested by the MacNichol-Mansfield transformation), $(\nu_1 - \nu_2)/\nu_{max} = C$, a constant, and HBW would be directly proportional to ν_{max}, *i.e.*, $(\nu_1 - \nu_2) = C\nu_{max}$.

By a generalization of the above expression for HBW, it can be shown that in a family of visual pigment spectra satisfying the aforementioned assumptions, any two spectra are related such that at equal ordinates, the abscissae vary in proportion to ν_{max}, *i.e.*, $\nu/\nu_{max} = \nu'/\nu'_{max}$. If we now take the primed symbols to refer to the template and the plain ones to a new spectrum to be generated, a corresponding frequency shift can be obtained at any given relative absorbance of the template. When expressed in the wavelength domain, the new abscissa at a given ordinate is

$$\lambda = \lambda'(\lambda_{max}/\lambda'_{max}) \qquad (1)$$

where λ_{max} is the desired new peak wavelength, while λ' and λ'_{max} are the corresponding abscissae of the template. Following evaluation of Eq. 1 for the discrete data pairs of the template spectrum (defined in this work between 310 and 630 nm at 5- and 10-nm increments), the result is a data set containing fractional wavelengths. To avoid the latter inconvenience, the data pairs are recalculated by linear interpolations to obtain spectral values at the nearest 5- and 10-nm integral wavelengths. The calculations were implemented on a personal computer, using a program coded in the BASIC computer language.

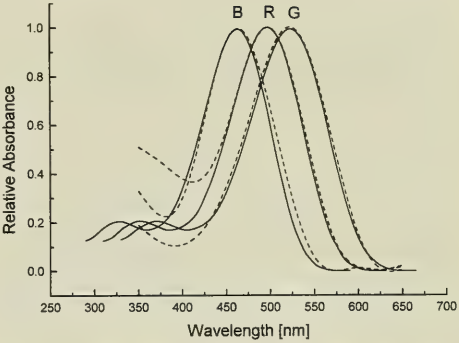

Figure 1. Comparison of experimental and modeled visual pigment spectra. The dashed curves show previously published experimental spectra from black sea bass photoreceptors: B, from single cones, λ_{max} = 464 nm; R, from rods, λ_{max} = 498 nm; G, from double cones, λ_{max} = 524 nm. The solid curves are calculated from the relative absorbance spectrum of bovine rhodopsin by the method described in the text. The modeled spectra were generated at the same λ_{max} as those of the experimental ones.

Figure 1 illustrates the fidelity with which this method reproduces previously published experimental absorbance spectra from the black sea bass (Singarajah and Hárosi, 1992). Although the experimental (dashed curves) and model (solid curves) spectra are in reasonable agreement for the α-bands, more serious deviations are apparent in the β-band regions. The discrepancies here, however, are probably due to artifacts in the experimental spectra rather than the transformation, even though the validity of the latter has not been proven for β-bands.

It is important to note that there is no "perfect" transformation capable of reproducing all features in all pigments. For example, the γ-band in visual pigment absorbance spectra changes not at all or only very little between pigments of disparate λ_{max}. In contrast to γ-band constancy, the α- and β-bands tend to shift further and further apart when λ_{max} is increased, whereas they approach each other more closely when λ_{max} is decreased. The crowding of the bands in shortwave-absorbing pigments may thus result in amplitude variations of the β-band, a subtlety that the current transformation cannot reproduce. Furthermore, in the case of the UV-absorbing visual pigments, even the α-bands resist simple modeling, because HBW variations in that spectral region become significantly nonlinear (Hárosi, 1994).

The model spectra generated in this study are considered to be sufficiently accurate for the intended purpose. The justification for this assessment comes from quantum-catch calculations carried out on visual pigments of the black sea bass. A comparison of computational results showed that total quantum catches and their ratios were nearly identical under a variety of conditions, whether the experimental or the generated spectra were used (*cf.* table II of Singarajah and Hárosi, 1992, and current Table II).

Quantal absorption, spectral irradiance, and water types

As previously discussed in Singarajah and Hárosi (1992), the basic premise for this modeling is that the photoreceptor cell is a quantum detector whose output is proportional to the rate of quantum catch (*i.e.*, the total number of quanta absorbed per unit time). Assuming the light stimulus to be the daytime downward irradiance, the needed information includes its amplitude and wavelength distributions for various water types and depths. First, the standard solar irradiance data of Moon (1940, table III) were used to generate quantal irradiances at sea level in quanta s^{-1} mm^{-2} nm^{-1}. Second, the transmitted irradiances were calculated using the tabulation on water properties by Jerlov (1968, table XX): his classifications were used unmodified as oceanic types I, IA, IB, II, and III, and coastal types 1, 3, 5, 7, and 9. Following Dartnall's (1975) suggestion, a "J" prefix was added to each of these labels to signify the source.

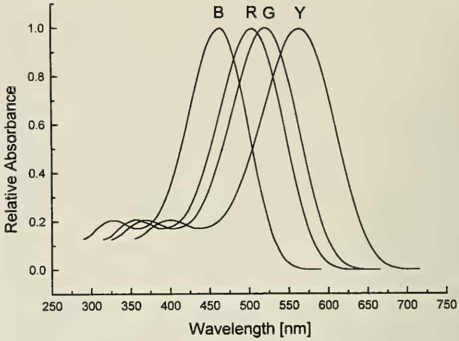

Figure 2. Model spectra for visual pigments of the sea raven. Curve labels are B, for the single cone pigment, λ_{max} = 463 nm; R, for rod pigment, λ_{max} = 505 nm; G, for one type of double cone pigment, λ_{max} = 522 nm; Y, for the second type of double cone pigment, λ_{max} = 565 nm.

Table II

Rates of total quantum catch by visual pigments, Q_t, in 10^{10} quanta s^{-1} mm^{-2} and their ratios as function of water type and depth (integrated in 5-nm steps from 280–700 nm)

Black sea bass (*Centropristis striata*)
Visual pigments: B, λ_{max} = 463 nm;
G, λ_{max} = 527 nm; R, λ_{max} = 498 nm
OCEANIC WATER TYPES:

Depth [m]	B	G	R	B/G	R/B	R/G
JIA						
100	287	491	583	.59	2.0	1.2
JIB						
10	3,766	12,124	10,525	.31	2.8	.87
50	852	1,837	1,969	.46	2.3	1.07
100	142	249	293	.57	2.1	1.18
200	4.3	6.5	8.2	.67	1.9	1.27
300	.14	.2	.26	.71	1.8	1.30
JII						
10	2,810	9,091	7,912	.31	2.8	.87
50	201	450	480	.45	2.4	1.07
100	8	15	17	.54	2.2	1.17
200	.014	.022	.027	.63	2.0	1.24
300	.000025	.000037	.000047	.67	1.9	1.28
COASTAL WATER TYPES:						
J3						
10	462	2,270	1,756	.20	3.8	.77
20	50	267	210	.19	4.2	.79
50	.09	.53	.43	.17	4.7	.81
J5						
10	91	626	433	.15	4.7	.69
20	2.5	21	14	.12	5.8	.68
50	.000095	.001	.00071	.09	7.5	.69

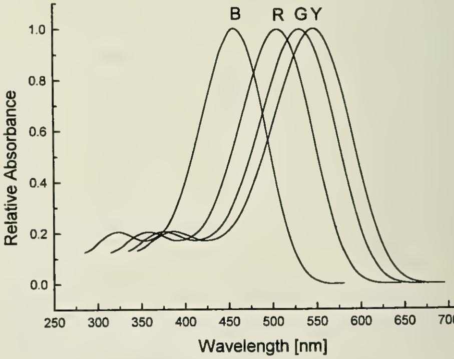

Figure 3. Model spectra for visual pigments of the adult winter flounder. Curve labels are B, for the single cone pigment, λ_{max} = 457 nm; R, for the rod pigment, λ_{max} = 506 nm; G, for one type of double cone pigment, λ_{max} = 531 nm; Y, for the second type of double cone pigment, λ_{max} = 547 nm.

Table III

Rates of total quantum catch by visual pigments, Q_t in 10^{10} quanta s^{-1} mm^{-2} and their ratios as function of water type and depth (integrated in 5-nm steps from 280–700 nm)

Sea raven (*Hemitripterus americanus*) Visual pigments: B, λ_{max} = 463 nm; G, λ_{max} = 522 nm; Y, λ_{max} = 565 nm; R, λ_{max} = 505 nm

OCEANIC WATER TYPES:

JIA

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
100	308	367	214	551	.84	1.73	.70	1.5

JIB

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	4,046	8,689	7,636	10,722	.47	1.14	1.89	1.2
50	915	1,362	888	1,909	.67	1.53	.97	1.4
100	152	186	109	278	.82	1.71	.72	1.5
200	4.6	4.9	2.6	7.6	.95	1.85	.57	1.6
300	.15	.15	.08	.24	1.01	1.91	.52	1.6

JII

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	3,019	6,526	5,688	8,061	.46	1.15	1.88	1.2
50	216	335	217	467	.65	1.55	1.00	1.4
100	8.6	11	6.5	16	.77	1.72	.76	1.5
200	.015	.016	.009	.0025	.89	1.85	.60	1.5
300	.000027	.000028	.000015	.000044	.96	1.91	.55	1.6

COASTAL WATER TYPES:

J3

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	497	1,618	1,522	1,856	.31	1.06	3.06	1.1
20	54	193	165	223	.28	1.17	3.05	1.2
50	.1	.4	.3	.5	.25	1.28	3.08	1.2

J5

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	98	439	474	471	.22	.93	4.81	1.1
20	2.7	14.9	14.9	15.8	.18	1.00	5.61	1.1
50	.0001	.0007	.0007	.0008	.14	1.10	6.66	1.1

Once the desired irradiances were available, the rate of quantal absorption by each pigment could be calculated from the axial *absorptance* of its receptor type, as follows. Given the peak axial optical density of a receptor *in situ* and the relative absorbance spectrum of its pigment, the absolute absorbance spectrum can be produced by scaling the relative spectrum to the peak axial optical density. If the latter spectrum is designated as $D(\lambda)$, the absolute absorptance spectrum is obtained as $A(\lambda) = 1 - 10^{-D(\lambda)}$. The product of $A(\lambda)$ with the spectral irradiance at a given wavelength (or a band of wavelengths) yields the rate of quantum flux absorption per unit area (for which the irradiance is defined). I used 1 mm^2 as "standard area" for the purpose of comparing the efficacy of the pigments. The rate of absorbed flux (quantum catch) was determined for 5-nm-wide bands of the spectrum from 280 to 700 nm, even though the data sets were commonly available for smaller portions of that range. The total quantum catch of a receptor type, Q_t, was obtained by piecewise integration over the entire spectrum (summation) of the 5-nm-wide quantum catches. All calculations were programmed in BASIC and carried out on a personal computer. The figures were plotted with the aid of ORIGIN (MicroCal Software, Inc., Northampton, MA 01060).

Results

Relative absorbance spectra were generated for the 11 known visual pigments of the three species for the λ_{max} listed in Table I. Figures 2 and 3 depict them for sea raven and winter flounder, respectively. Similar spectra were already illustrated (Fig. 1) for the black sea bass, though

Table IV

Rates of total quantum catch by visual pigments, Q_t in 10^{10} quanta s^{-1} mm^{-2} and their ratios as function of water type and depth (integrated in 5-nm steps from 280–700 nm)

Winter flounder (*Pseudopleuronectes americanus*) Visual pigments: B, λ_{max} = 457 nm; G, λ_{max} = 531 nm; Y, λ_{max} = 547 nm; R, λ_{max} = 506 nm

OCEANIC WATER TYPES:

JIA

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
100	148	149	120	450	.99	1.25	.81	3.0

JIB

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	1,870	4,006	3,838	8,853	.47	1.04	2.05	2.2
50	431	581	490	1,568	.74	1.19	1.14	2.7
100	73	76	61	227	.95	1.25	.84	3.0
200	2.2	1.9	1.5	6.2	1.17	1.29	.66	3.2
300	.07	.06	.04	.2	1.27	1.31	.60	3.3

JII

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	1,392	3,007	2,871	6,659	.46	1.05	2.06	2.2
50	101	143	120	384	.71	1.19	1.19	2.7
100	4.0	4.6	3.7	13.4	.88	1.25	.91	2.9
200	.007	.007	.005	.02	1.07	1.29	.72	3.2
300	.000013	.000011	.000008	.00004	1.18	1.31	.65	3.3

COASTAL WATER TYPES:

J3

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	211	773	762	1,546	.27	1.01	3.61	2.0
20	22	91	87	186	.24	1.05	3.94	2.0
50	.04	.18	.17	.38	.21	1.09	4.43	2.1

J5

Depth [m]	B	G	Y	R	B/G	G/Y	Y/B	R/G
10	40	217	226	394	.18	.96	5.70	1.8
20	1.0	7.4	7.5	13.2	.14	.99	7.47	1.8
50	.000035	.00037	.00036	.00067	.09	1.02	10.4	1.8

with slightly different λ_{max} values. Quantum-catch calculations were carried out for all receptor types, using generated spectra, under the conditions of several water types and depths. Information about the habitats of the species may be recapitulated as follows.

As noted in Singarajah and Hárosi (1992), the black sea bass is a widely distributed species from southern Massachusetts to northern Florida and from bays and sounds to the Georges Bank region of the Atlantic Ocean. These fishes inhabit shallow waters of a few meters to well over 100 m in depth. They are primarily bottom feeders and are caught in large numbers at depths of 50–150 m.

Sea ravens are also widely distributed along the Atlantic coast of North America from the Gulf of St. Lawrence to as far south as the Chesapeake Bay, preferring cooler waters. They are found all around the Gulf of Maine, in deeper harbors of Massachusetts Bay, to the shoals of Georges Bank and around Cape Cod. Sea ravens are reportedly caught only over rocky, hard sand bottoms from a few meters down to about 200 m, with their usual range extending to only about 100 m (Bigelow and Schroeder, 1953).

The winter flounder is a nonmigratory flatfish with wide distribution along the Atlantic coast of North America from the north shore of the Gulf of St. Lawrence to as far south as North Carolina and Georgia. It is very abundant and of great importance to New England commercial fishing. Winter flounder can be found in shallow inshore waters as well as in the Gulf of Maine and on Georges Bank, in bays and harbors around Cape Cod, and in somewhat deeper water of the Nantucket Shoals region. The species is sedentary and prefers soft, sandy or muddy,

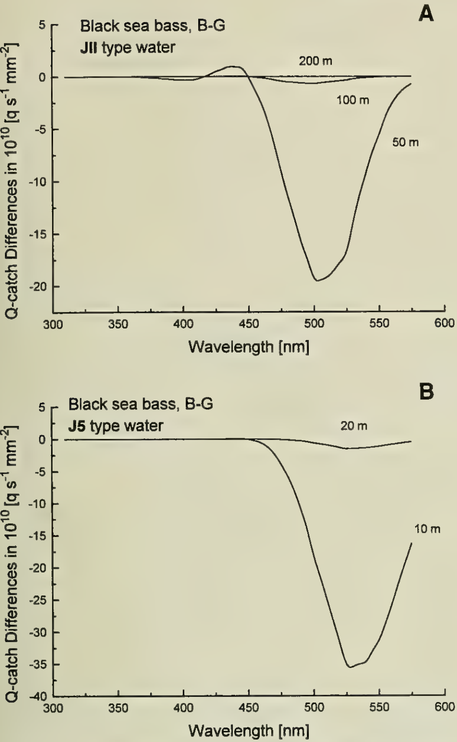

Figure 4. Regional quantum-catch difference spectra in 5-nm-wide piecewise approximation. Black sea bass receptor types, B–G. (A) JII type water at depths of 50, 100, and 200 m. (B) J5 type water at 10 and 20 m.

bottoms. Its vertical range extends from a few meters to 140 m as the deepest record known. These flounder are plentiful at 20 to 40 m in Cape Cod Bay and can be taken between 50 and 90 m on Georges Bank (Bigelow and Schroeder, 1953; Backus, 1987).

The selection of water types was based on published tabulation (Clarke, 1939) of field measurements (Oster and Clarke, 1935) carried out on natural bodies of water in the habitats of interest. The procedure was to convert the average "transmissive exponent," k (also called "absorption coefficient"), determined in three wavelength ranges, to "irradiance transmittance" (%/m) values, and then correlate them with those of table XX of Jerlov (1968) for water types. The identification of water types with locations made in this manner was as follows: Nantucket Shoals, JIA; Georges Bank, JII; Vineyard Sound and Buzzards Bay, J3; Woods Hole Harbor, J5.

Rates of total quantum catch by the receptor types of the three species in various water types and depths are listed in Tables II, III, and IV. The results show absolute rates of pigment absorption and their ratios for each species. Although the water types of interest were JII and J5, calculations on others types, some of which were included in the tables, display the effect of water quality on visual function. For example, an inspection of data in Table II indicates the following. The blue pigment at 100 m may catch 287 photons (in 10^{10} quanta s^{-1} mm^{-2}) in JIA water; the figure drops to 142 in JIB and to 8 in JII water. And, although the absolute rates fall 1–2 orders of magnitude, the B/G quantum-catch ratios show only minor changes (0.59, 0.57, and 0.54). This trend appears to be similar in coastal waters, even though quantum-catch rates are reduced significantly. Thus, at 20-m depth in J3 and J5 type water, although the absolute rates are 50 and 2.5, the B/G ratios change from 0.19 to 0.12, respectively.

Integrated and regional quantum catches

Although total quantum catch might be a useful parameter to gauge the overall suitability of a photoreceptor type to interact with certain photic environments, it is not a sensitive measure of variations in narrow spectral regions. In comparing narrow-band responses between receptor types, the resolution may be limited by the availability of data points. Although interpolation of data to steps of 1 nm is feasible, it was unnecessary, because spectral absorptance averaged over 5-nm-wide segments of the spectrum adequately approximated regional quantum-catch variations. This point is illustrated with the quantum-catch difference spectra plotted in Figures 4–6.

Discussion

Inspection of the data presented in Tables II–IV reveals some unexpected results. For instance, the rates of quantum catch by photoreceptor types within each retina of each species studied appear to be similar. This means that the sensory pigments, at the given outer segment densities and at 1 mm^2 cross section, have nearly equal abilities for photon capture throughout the habitats. A comparison of rod and cone spectral sensitivity of human vision showed the rod function to be higher by 1–3 orders of magnitude at certain wavelengths (Wald, 1945). On the basis of such studies (see also Pirenne, 1962), one might expect the photon capture of rods to surpass that of cones significantly. Because that is clearly not the case in the species investigated here, any increase in overall gain of rod function must be achieved elsewhere. There are several possible ways to increase rod sensitivity—for one, by setting signal amplification within the receptors high; for others, by increasing the rod-to-cone ratio (*e.g.*, this ratio is about 20:1 in the human retina and 15:1 in that of

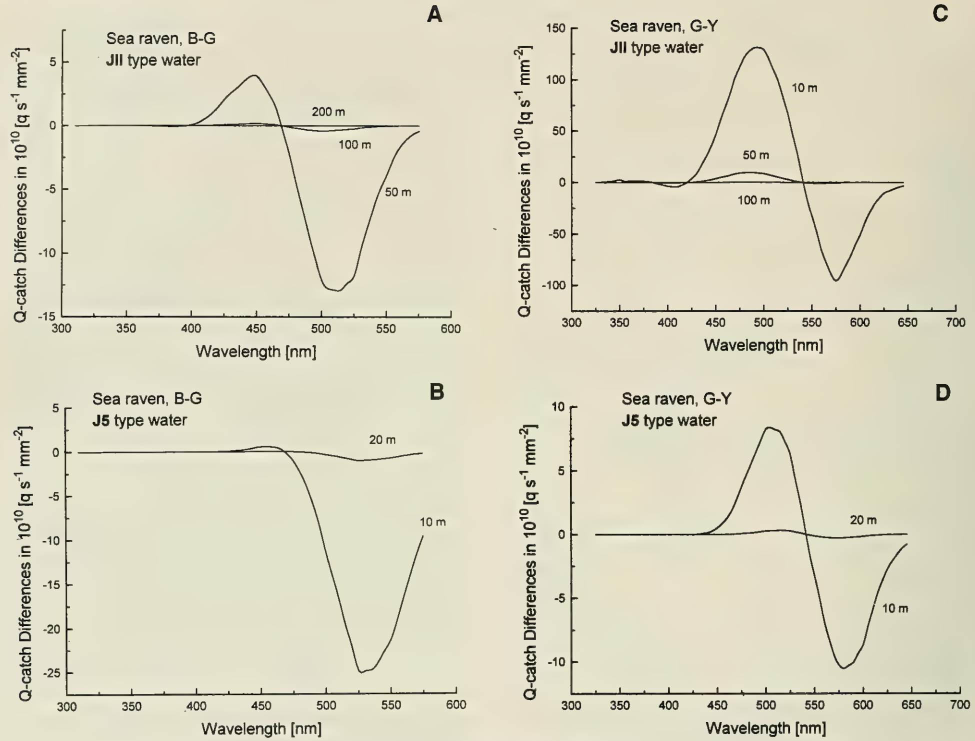

Figure 5. Similar to Figure 4, except for sea raven photoreceptors. (A) B–G for JII type water at depths of 50, 100, and 200 m. (B) B–G for J5 type water at 10 and 20 m. (C) G–Y for JII type water at 10, 50, and 100 m. (D) G–Y for J5 type water at 10 and 20 m.

goldfish); pooling inter-receptor rod signals; or elevating the rods' share in the light capture area by retinomotor movements. Quantum catches among the cones in each retina appear to be even more closely matched than those with respect to the rods. This appears to be not only reasonable, but also necessary, if cone output signals are being compared in wavelength discrimination.

In view of its photopigment characteristics, the visual system of the black sea bass is tuned to the blue-green (460–530 nm) region of the spectrum. Given that males have been observed to develop bright blue coloration prior to spawning, the detection of blue light might indeed be important for this species. As indicated by the quantum-catch difference spectra in Figure 4A, blue-green discrimination is functional to at least 50 m in JII water. In coastal waters, where blue light is greatly diminished, this species becomes essentially monochromatic. Nevertheless, its vision should continue to operate well with its large double cones that contain the green-absorbing pigment (Fig. 4B). Although in blue-deprived waters the G and R receptors of the fish should continue to capture photons at all depths considered, it is not known whether they can also participate in yellow-green discrimination.

The other two species studied here have a clear-cut advantage over the black sea bass by virtue of an extra cone type whose photosensitive pigment has peak absorption in the yellow-red spectral region. The sea raven appears to have active blue-green discrimination not only in clear ocean water (JII) to at least 100 m depth (Fig. 5A), but even in coastal waters (J5) to as deep as 10 m or so (Fig. 5B). But the striking feature of the photoreceptors in this species is their superbly balanced responsiveness in the red-green region of the spectrum (Fig. 5C, D). Although the scale is unfavorable to show the quantum-catch difference spectrum for J5 water at 20 m (Fig. 5D), it is as "balanced" as the one at 10 m, even though it is about

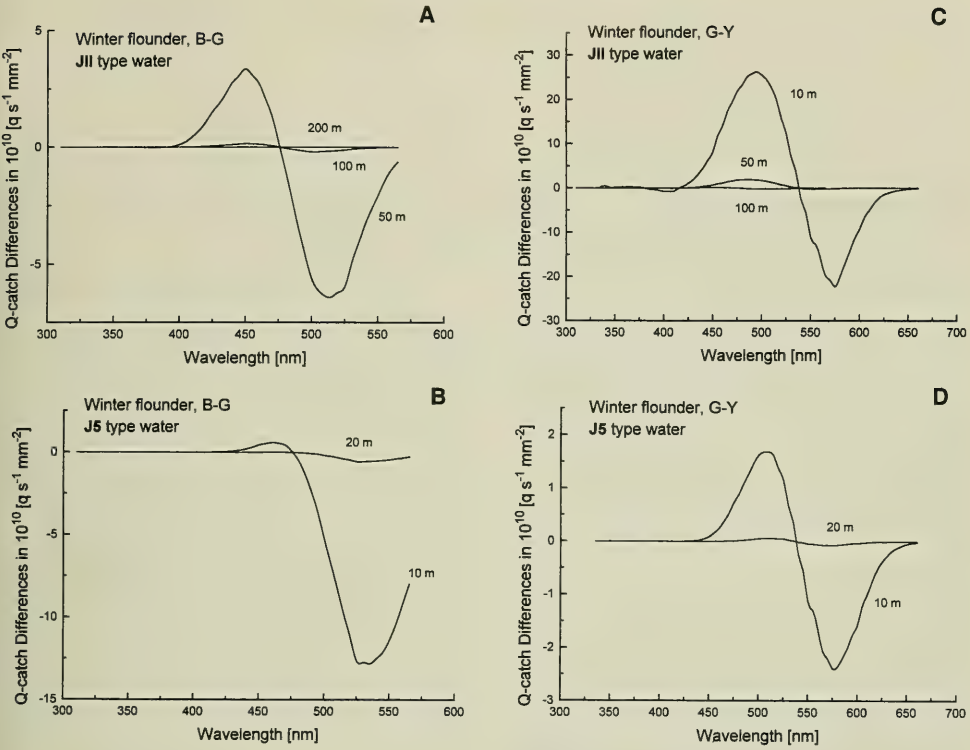

Figure 6. Similar to Figure 4, except for winter flounder photoreceptors. (A) B–G for JII type water at depths of 50, 100, and 200 m. (B) B–G for J5 type water at 10 and 20 m. (C) G–Y for JII type water 10, 50, and 100 m. (D) G–Y for J5 type water at 10 and 20 m.

30-fold smaller in absolute magnitude (*cf.* Table III). This analysis indicates that red-green discrimination might be important to the species. The color of sea raven is reported to be greatly variable—from blood red, to brown, to yellow-orange. Many are plain colored. But their eggs have a yellow-to-amber hue and the females practice the interesting habit of depositing their eggs preferentially at the branches of two species of sponge, which are of pinkish and yellowish color (Bigelow and Schroeder, 1953; Collins and MacNichol, 1984). In view of these facts, the results of the analysis are reassuringly coherent.

For winter flounder (Fig. 6A–D), perhaps the most intriguing aspect of the results is the similarity of their quantum-catch difference spectra to those of the sea raven. To be sure, each of these two species has three cone and a rod pigment and their λ_{max} are quite similar and almost "human-like." Both species also inhabit similar photic environments, but they have very different habits. Habit differences notwithstanding, their receptor quantum catches are alike. The body color of winter flounder varies according to the bottom on which they lie. It may be muddy or slightly reddish-brown, olive green, or nearly black. The adult winter flounder is limited by its small mouth to a diet of small invertebrates and fish fry. The winter flounder spawns on sandy bottom, often in shallow water or as deep as 50–80 m on Georges Bank, and lays eggs that sink to the bottom, where they may stick together and form clusters (Bigelow and Schroeder, 1953). Thus, although the flounder does not share the sea raven's egg-laying needs, both species are equipped with photoreceptors capable of very similar "color vision." Of course, it is not known whether both species could perform color discrimination tasks equally. Nonetheless, if visual pigments are the primary determinants of this sensory modality, the two species must have very similar capabilities. And although the winter flounder's habits are different from those of the sea raven, they, too, may put their color vision to good use—for example, in the capture of prey.

In order for the black sea bass to improve blue-green discrimination, the "blue catch" must be increased (Fig. 4A–B). This improvement could be implemented by increased length of the single cone outer segments, increased area of light collection for single cones, or increased numbers of single cones compared to double cones. The question of why this species has no red receptor cannot be answered directly. It may be, however, that by being a bottom-dweller, the fish seeks out longwave-deprived environments, where disadvantages stemming from the lack of red receptors are minimized. Further compensation may come from having a pigment with a λ_{max} equal to 527 nm in its large double cones, thus providing good sensitivity in the yellow, even in the absence of wavelength discrimination. The latter suggestion is based on results plotted in Figs. 4B, 5B, and 6B. Comparison of the three species in J5 water at 10 m, a depth at which black sea bass are routinely being trapped in Woods Hole Harbor, reveals that in the range of 525 to 575 nm, this species has more quantum catch than either the raven or the flounder.

How many receptor types a retina should utilize and how the λ_{max} of the pigments should be picked are questions that seem to have no simple answer. The approach is probably that of a minimalist—*i.e.,* the fewest possible number still commensurate with the habits and habitats of the species. The visual system of the black sea bass may serve as an example of retinas having three types. The exact λ_{max} values are probably not critical. What appears to be more important is maintaining a balance of quantum catches between the receptor types, especially those that feed into wavelength-discrimination channels. The sea raven and winter flounder might be regarded as typical for four-receptor-type visual systems. The similarity in quantum catches in these two species, however, remains enigmatic, indicating the need for further investigation.

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Expression Sites of Two Byssal Protein Genes of *Mytilus galloprovincialis*

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Abstract. Mussels form byssal threads that can attach tenaciously to wet and irregular surfaces. The byssus consists of a fibrous collagenous core, and at least two types of polyphenolic proteins surround it. One of these proteins, designated Mgfp-1, coats the collagenous core; the other, designated Mgfp-2, is the major component of the terminal adhesive plaque of byssal threads. Both proteins contain 3,4-dihydroxyphenylalanine (DOPA) in their primary sequences. In this study, the sites of expression of the genes encoding the polyphenolic proteins were investigated in *Mytilus galloprovincialis.* By northern blot analysis, we found that the expression of both genes is foot-specific. Northern blot analysis of RNA isolated from the distal end and the remaining proximal portion of the foot indicated that the Mgfp-2 gene is expressed primarily in the distal part, whereas Mgfp-1 expression occurs in both parts. *In situ* hybridization indicated that the Mgfp-1 gene transcript is localized in the accessory gland along the ventral groove of the foot, and the Mgfp-2 gene transcript is localized in the phenol gland near the foot apex. Thus, it was shown that tissues expressing Mgfp-1 and Mgfp-2 are located around the ventral groove in an arrangement appropriate for byssus formation.

Introduction

Marine sessile invertebrates such as mussels and barnacles adhere tightly to wet and irregular surfaces in the sea. They have evolved specific adhesive mechanisms to resist the impact of waves. For example, barnacles secrete cement proteins (Saroyan *et al.,* 1970) and mussels secrete byssus threads. These substances act as glues that becomes insoluble after secretion in water and attach the animal to the substrate.

The byssus of mussels, which has a high bonding strength (Allen *et al.,* 1976), is a bundle of threads, each consisting of a fibrous collagenous core coated by adhesive proteins. The protein components of the byssus have been studied extensively in *Mytilus edulis* (Waite, 1987, 1992, for reviews). A major component, foot protein 1 (fp-1), is an adhesive 130-kDa protein containing a high proportion of 3,4-dihydroxyphenylalanine (DOPA) residues (Waite and Tanzer, 1981; Waite, 1983). Its primary sequence exhibits tandem repeats of the decapeptide motif Ala-Lys-Pro-Ser-Tyr-diHyp-Hyp-Thr-DOPA-Lys, where diHyp and Hyp are 3,4-dihydroxyproline and 4-hydroxyproline, respectively (Waite *et al.,* 1985; Taylor and Waite, 1994). Another component, foot protein 2 (fp-2), is also a DOPA-containing protein that is structurally unrelated to fp-1. It is a plaque matrix protein of 42–47 kDa in size; it contains a smaller proportion of DOPA residues than fp-1 and is rich in the disulfide-containing amino acid cystine (Rzepecki *et al.,* 1992).

Partial sequences of cDNA and genomic DNA encoding fp-1 have been reported in *M. edulis* (Strausberg *et al.,* 1989; Filpula *et al.,* 1990; Laursen, 1992). Recently, cDNA sequences encoding the whole coding region of the two foot proteins of *M. galloprovincialis,* another mussel species closely related to *M. edulis,* have been determined (Inoue and Odo, 1994; Inoue *et al.,* 1995a). The cDNA of *M. galloprovincialis* fp-1 (Mgfp-1) encodes 62 repeats of the same decapeptide motif as that of *M. edulis,* but lacks the hexapeptide motif (Inoue and Odo, 1994; Inoue *et al.,* 1995b). Sequencing of the *M. galloprovincialis* fp-2 (Mgfp-2) cDNA has shown that the Mgfp-2 protein

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; fp-1, foot protein-1; fp-2, foot protein-2; Mg, *Mytilus galloprovincialis;* Me, *Mytilus edulis.*

consists mainly of repeats resembling those of epidermal growth factor (EGF) (Inoue *et al.,* 1995a).

Still, many questions remain unresolved. The phenol gland is an apparent site of fp-1 synthesis and storage, so the apparent localization of fp-2 to the phenol gland is a little ambiguous (Rzepecki *et al.,* 1992; Waite, 1983; Benedict and Waite, 1986). In this study, we examined expression sites of the Mgfp-1 and Mgfp-2 genes by northern blots and *in situ* hybridization. We found that tissues expressing the Mgfp-1 and Mgfp-2 genes were located along the ventral groove of the foot in an arrangement appropriate for byssus formation.

Materials and Methods

Isolation of total RNA

Mussels (*M. galloprovincialis*) of about 6 cm in shell length were purchased from an aquaculture company in Rikuzen-Takada, Iwate prefecture, Japan. The foot, mantle, gill, and adductor muscle were isolated from mussels, and about one quarter of the distal part, which contains the phenol gland, and the remainder of the foot, which contains the accessory gland, were separated with a scalpel. Total RNA was extracted by using the Total RNA Separator Kit (Clontech Laboratories, Palo Alto, CA).

Northern blot hybridization

Ten micrograms of total RNA obtained from mussel tissues was electrophoresed on a 0.8% agarose gel containing 2.2 *M* formaldehyde and 0.02 *M* 3-(*N*-morpholino) propanesulfonic acid and transferred onto a nylon membrane (Hybond-N$^+$, Amersham). Hybridization was performed with cDNA encoding the whole coding region of Mgfp-1 and Mgfp-2 (Inoue and Odo, 1994; Inoue *et al.,* 1995) labeled with [α-^{32}P]dCTP in accordance with the instructions for the Random Primer DNA Labeling Kit (Takara Shuzo Co., Ltd. Kyoto, Japan.) The hybridization buffer contained 5× standard saline citrate 5× Denhardt's mediums, 0.1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate, and 100 μg/ml sonicated salmon sperm DNA. After prehybridization, denatured probe was added and the nylon membrane was incubated at 60°C for 16 h. The nylon membrane was washed to a final stringency of 0.1× standard saline citrate at 60°C. The radioactive signals were detected by exposing the membrane for 1 h to X-ray film at −80°C with an intensifying screen.

In situ *hybridization*

M. galloprovincialis foot was fixed with 4% (w/v) paraformaldehyde dissolved in phosphate-buffered saline (PBS) at 4°C for 4 h. The fixative was replaced with 30% sucrose dissolved in PBS at 4°C for 2 h. Then the foot was embedded in OTC compound (Miles, Inc.), frozen at −20°C, and cut into 13-μm thick sections. Sections were attached to slide-glass coated with gelatin, immediately dried for 1 h, and further dried in an oven at 45°C for 2 h. Digoxigenin-labeled sense- and antisense-RNA probes were prepared according to the instructions for the DIG RNA labeling kit (Boehringer Mannheim Biochemica). Sense and antisense probes were synthesized with T3- and T7-RNA polymerases, and full-length cDNA of Mgfp-1 and Mgfp-2 was subcloned into the *Eco*RI-*Xho*I sites of a plasmid vector, BluescriptIISK+ (Stratagene), as a template. The template plasmids were digested with *Hin*dIII/*Spe*I and *Cla*I/*Spe*I before transcription by T3- and T7-RNA polymerases, respectively. RNA probes were degraded into about 150 bases by alkaline treatment at 60°C for 8 and 2.8 min, respectively. Hybridization and washing were performed, as described by Yokouchi *et al.* (1991). Detection was carried out according to the instructions of the DIG Detection kit (Boehringer Mannheim Biochemica).

Results

Northern blot hybridization

To identify tissues expressing the Mgfp-1 and Mgfp-2 genes, northern blot hybridization was performed using RNA from major organs—*i.e.,* foot, gill, mantle, and adductor muscle. The Mgfp-1 and Mgfp-2 genes were detected only in the foot, not in other organs (Fig. 1). To

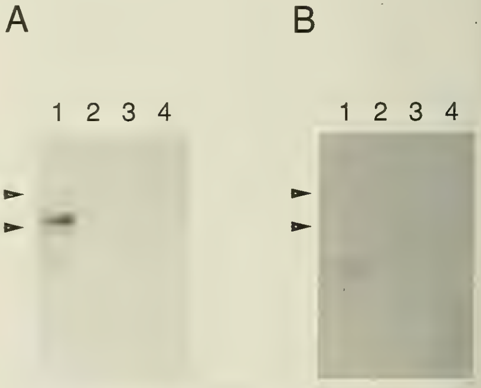

Figure 1. Northern blot hybridization of RNA extracted from *Mytilus galloprovincialis* tissues. Ten micrograms of total RNA was electrophoresed on a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with ^{32}P-labeled cDNA encoding the whole coding region of Mgfp-1 and Mgfp-2. (A) Mgfp-1 sequence as a probe; (B) Mgfp-2 sequence as a probe. 1, foot; 2, mantle; 3, muscle; 4, gill. Arrowheads indicate the position of 18S and 28S rRNA.

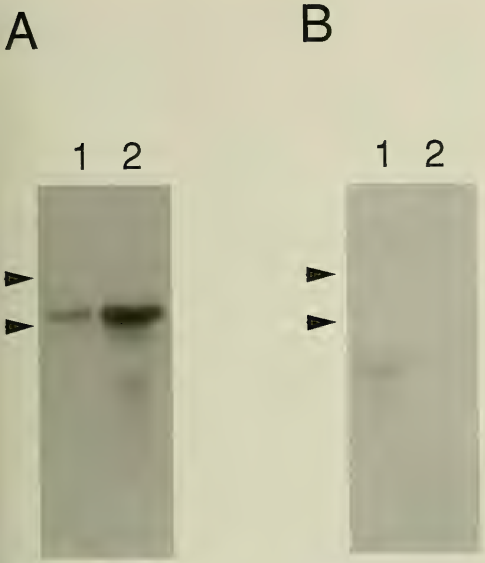

Figure 2. Northern blot hybridization on RNA extracted from the distal part and the rest of the foot of *Mytilus galloprovincialis.* Ten micrograms of total RNA was electrophoresed on a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with ^{32}P-labeled cDNA encoding the whole coding region of Mgfp-1 and Mgfp-2. (A) Mgfp-1 sequence as a probe; (B) Mgfp-2 sequence as a probe. 1, the distal part of the foot; 2, the remainder of the foot. Arrowheads indicate the position of 18S and 28S rRNA.

estimate the expression sites of the two genes in the foot, RNA was isolated separately from the distal end and from the remainder of the foot and was also analyzed by northern blotting. The distal end of the foot consisted mainly of the phenol gland, but included a small fraction of the accessory gland, because there is a hairpin turn of the accessory gland interdigitated with the underlying phenol gland (Waite, 1992). As a result, Mgfp-1 mRNA was detected in both the distal end and the remainder of the foot, whereas Mgfp-2 mRNA was detected primarily in the distal end and was undetectable in the remainder of the foot. Moreover, the level of Mgfp-1 mRNA accumulation was higher in the remainder of the foot than in the distal end (Fig. 2). These results indicated that the Mgfp-1 and Mgfp-2 genes were expressed at different sites in the foot. The length of Mgfp-1 cDNA was about 2.5 kb (Inoue and Odo, 1994). This size was consistent with the position of the bands detected in Figures 1A and 2A. The length of Mgfp-2 cDNA was about 1.5 kb (Inoue *et al.,* 1995), and this size was also consistent with the position of the bands detected in Figures 1B and 2B. Mgfp-1 mRNA accumulated so much that there were degraded bands below the major band (Figs. 1A, 2A).

In situ *hybridization*

The foot is an organ that has many functions. It contains various types of tissues and glands (Waite, 1992). To determine exact sites expressing the Mgfp-1 and Mgfp-2 genes in the foot, *in situ* hybridization was performed. The foot was dissected longitudinally and the sections were hybridized with sense- and antisense-RNA probes. It was shown that the Mgfp-1 transcripts accumulate in cells around the ventral groove of the foot corresponding to the accessory gland, and that the transcript were barely detectable in the phenol gland (Fig. 3). The Mgfp-2 transcripts, in contrast, were detectable only in the distal phenol gland (Fig. 4).

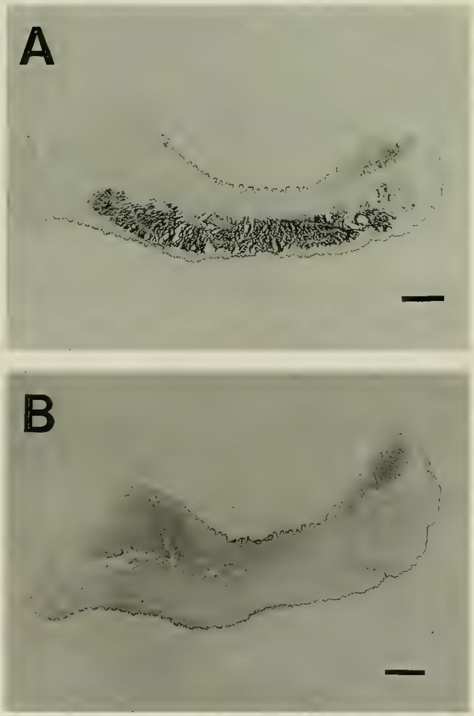

Figure 3. *In situ* hybridization of the Mgfp-1 gene transcripts in the foot of *Mytilus galloprovincialis.* Hybridization was performed using the digoxigenin-labeled antisense-probe (A) and sense-probe (B) RNAs. Bar = 1 mm.

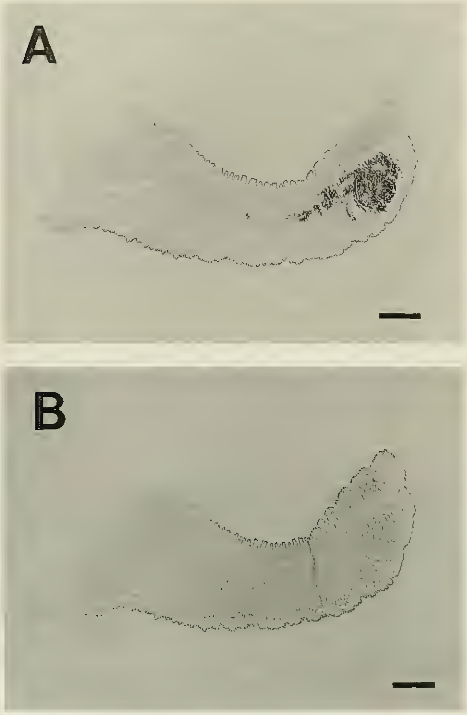

Figure 4. *In situ* hybridization of the Mgfp-2 gene transcripts in the foot of *Mytilus galloprovincialis.* Hybridization was performed using the digoxigenin-labeled antisense-probe (A) and sense-probe (B) RNAs. Bar = 1 mm.

Discussion

Around the ventral groove of the mussel foot are various glands—such as the accessory gland, the phenol gland, and the collagen gland (Waite, 1992)—from which thread-forming materials are secreted. In this study, we determined the expression sites of two foot proteins of *M. galloprovincialis,* Mgfp-1 and Mgfp-2. We showed that Mgfp-1 and Mgfp-2 mRNA accumulated in the foot and not in other tissues (Fig. 1). We also showed that the two types of mRNA accumulated at different sites of the foot (Fig. 2). By *in situ* hybridization analysis of the foot, Mgfp-1 mRNA was found in the accessory gland around the ventral groove (Fig. 3), and Mgfp-2 mRNA was found in the phenol gland (Fig. 4).

It is known that Mgfp-1 forms a protective coat on the collagenous core of byssal threads (Brown, 1952; Pujol, 1967; Vitellaro-Zuccarello, 1981), and that the collagenous core of the threads and the polyphenolic protein protecting it are secreted by the collagen gland and the accessory gland, respectively (Brown, 1952; Pujol, 1967; Vitellaro-Zuccarello, 1980, 1981). The distal plaque matrix of byssal threads consists of Mefp-2 (Rzepecki *et al.,* 1992). The plaque matrix is formed by secretion from the phenol gland at the distal depression (Rzepecki *et al.,* 1992), and Mefp-2 is detectable only in extracted foot tip (Waite, 1992).

By comparing the location of the proteins and the sites of accumulation of the mRNA for the proteins, we conclude that the expression sites are located around the ventral groove in an arrangement appropriate for byssus formation (Fig. 5). Since Mgfp-2 was detected exclusively in the phenol gland in the previous study (Rzepecki *et al.,* 1992), the phenol-gland-specific expression of Mgfp-2 gene was expected (Fig. 4). However, the very low level expression of Mgfp-1 in the phenol gland was unexpected (Fig. 3) because it has been reported that an antibody to Mefp-1 binds strongly to the phenol gland as well as to the accessory glands (Benedict and Waite, 1986) and that the phenol gland is an apparent site of Mefp-1 storage (Waite, 1983; Benedict and Waite, 1986). Furthermore, Mefp-1 has been isolated from dissected phenol glands as well as from the accessory gland (Waite, 1983, 1992). We thus interpret our results to suggest that the Mgfp-1 gene is transcribed in the accessory gland and the translated protein is delivered to and stored in the phenol gland. We also postulate that Mgfp-1 and Mgfp-2 are synthesized as raw materials in the accessory and phenol glands, respectively, and that Mgfp-1 is stored in both the accessory and phenol glands, but Mgfp-2 only in the phenol gland. Mussels may then secrete each protein as needed when thread is formed. We also have another interpretation for the

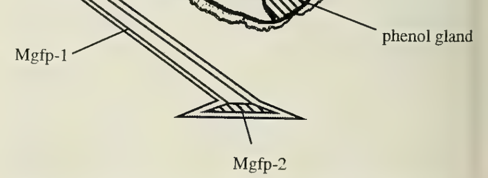

Figure 5. Schematic diagram of the foot and byssus of *Mytilus galloprovincialis* in longitudinal section.

presence of Mefp-1 in the phenol gland region. The accessory gland makes a hairpin turn around the distal depression, and the phenol gland is just before it (Waite, 1992); the two glands may have an overlap there. We suggest that phenol gland dissections include an overlap between the accessory and phenol glands, so an antibody to Mefp-1 binds to the phenol gland and Mefp-1 can be isolated from dissected phenol gland.

Mgfp-2 is a protein consisting mainly of EGF-like repeats (Inoue *et al.,* 1995). Generally, EGF-like proteins promote cell-to-cell interactions (Massagué, 1990). It is, therefore, interesting to ask whether Mgfp-2 has a function in addition to its structural role in the plaque matrix. In this study, we found that the Mgfp-2 gene is expressed primarily in a limited part of the foot—*i.e.,* at the distal phenol gland (Fig. 4). This observation strongly suggests that Mgfp-2 has a specialized function, to form the matrix of the secreted protein, but does not act to promote cell-to-cell interaction.

Acknowledgments

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To be Stiff or to be Soft—the Dilemma of the Echinoid Tooth Ligament. I. Morphology

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Abstract. The morphology of the tooth ligament of the echinoid *Eucidaris tribuloides* was investigated. The tooth ligament connects the middle part of the rod-shaped tooth to the jaw. It consists of collagen and microfibrils that are synthesized by fibroblasts in the aboral growth zone of the tooth. Histochemical staining showed that the tooth ligament contains highly sulfated acid proteoglycans that seem to connect the collagen fibrils. We did not find any cell bodies in the ligament, but we observed a small number of cell processes. Dense vesicle cells and supporting cells in the jaw near the ligament send these processes among the collagen fibrils. Dense vesicle cells have connections to a newly described jaw nerve. Supporting cells possess a unique podocyte-like filtration structure that probably serves for selective pinocytosis. In the oral region, where the working part of the tooth protrudes from the jaw, collagen fibrils swell and disappear. Sometimes phagocytosis of collagen fibrils could be observed. Autoradiographic data indicate that the collagen of the tooth ligament is not subject to quick turnover. The tooth ligament resembles a catch connective tissue that can change its mechanical properties under nervous control.

Introduction

Regular sea urchins possess a masticatory apparatus, the lantern of Aristotle. The lantern consists of five jaws each holding one tooth (Fig. 1). The tooth can be divided into three regions along its longitudinal axis. Each tooth is connected to the jaw only along its middle part by a ligament (Fig. 2). The oral part is the working part and is steadily worn away during use. To compensate for this shortening of the tooth, new tooth material is constantly synthesized in the aboral soft growth zone, the plumula (Fig. 3). During growth the tooth slides slowly along the jaw at about 1–1.5 mm/week (Holland, 1965; Märkel, 1969).

The lantern is a powerful instrument: some sea urchins such as *Eucidaris tribuloides* and *Diadema setosum* scrape the surface of rocks and hard corals to feed on algae and encrusting organisms, and thus their teeth are subject to strong forces (Märkel *et al.*, 1977). The need to withstand this pressure during feeding dictates that the teeth be fastened tightly; on the other hand, the tooth ligaments have to be soft enough to allow slow shift of the teeth during growth.

Rodents face a similar dilemma. They solve it by remodeling of collagen. A number of cells are dispersed along the periodontal ligament that connects tooth and jaw. These cells can synthesize and degrade collagen at the same time (Beertsen and Everts, 1977; Ten Cate *et al.*, 1976).

Whereas the periodontal ligaments of mammalian teeth are well investigated, the tooth ligament of echinoids has never been studied. The present investigation provides the first thorough morphological data, including the important aspects of collagen synthesis, collagen degradation, and innervation. Our results indicate that sea urchins solve their dilemma in a different way from that of rodents. We hypothesize that the tooth ligament is a catch connective tissue. Evidence for this hypothesis will be provided in the succeeding paper (Birenheide *et al.*, 1996), which describes the mechanical properties of the tooth ligament and their changes under nervous control.

Some preliminary data have been presented as a conference paper (Birenheide, 1990).

Materials and Methods

Specimens of *Eucidaris tribuloides* (Lamarck, 1916) were obtained from aquarium supply companies. They

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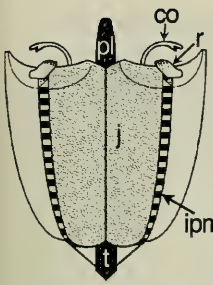

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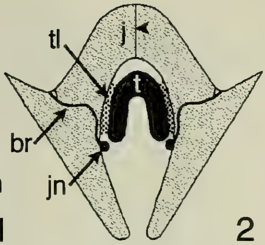

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Figure 1. Schematic view of the lantern of *Eucidaris tribuloides.* The lantern is oriented with the tips of the teeth at the bottom and the plumulae at the top. One jaw (j) is stippled and its corresponding tooth (t) is black. co: compass, ipm: interpyramidal muscle, pl: plumula of tooth, r: rotula.

Figure 2. Schematic cross section of a jaw and a tooth of *Eucidaris tribuloides.* br: branch of jaw nerve penetrating jaw, j: jaw, jn: jaw nerve, t: tooth, tl: tooth ligament, arrowhead: suture where two halves of one jaw are connected.

were kept for months in aquaria with artificial seawater and fed algae and aquarium fish food. Appropriate pieces of the lantern were dissected and immediately fixed. Routine fixation was for 18 h in 5% glutaraldehyde in 0.05 *M* cacodylate buffer (pH 7.8) containing 5 m*M* $CaCl_2$; postfixation was in 1% osmium tetroxide including 0.8% $K_3Fe(CN)_6$ in the same buffer. This method will be called GO-fixation. Some small fibrils, especially in the plumula, were best fixed in a 1% osmium tetroxide solution in 0.05 *M* cacodylate buffer saturated with potassium pyroantimonate. This fixation will be called osmium fixation.

To visualize hyaluronic acid, 1% low molecular weight tannin was added to the glutaraldehyde solution during routine fixation (Singley and Solursh, 1980). For visualization of proteoglycans, 0.1% Safranin (CI-No. 50240) was added to the glutaraldehyde solution and 0.00125% Safranin was added to the postfixation solution (Shepard and Mitchell, 1976). Sucrose was added to all fixation solutions until the solutions had the same osmolarity as seawater. After fixation, specimens were dehydrated in an ethanol series and embedded in araldite. The blocks were abraded with emery paper to expose the calcite, then decalcified and reembedded (Märkel and Röser, 1983). With this method the calcite of the ossicles is replaced by featureless araldite; therefore our micrographs show apparently empty spaces whose shapes correspond exactly to the calcite of jaw and tooth. Semithin sections of 1-μm thickness were stained with 1% crystal violet at 60°C. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in an electron microscope, either a Zeiss 9S-2 or a Hitachi H-300.

For light microscopical histochemistry, fixation was in buffered formalin for several days. Specimens were decalcified in 1% EDTA solution and embedded in paraffin. Sections were dewaxed and stained with routine methods (Humason, 1979). Staining methods with alcian blue in critical electrolyte concentration are described by Scott and Dorling (1965).

For autoradiographic experiments, specimens of *Eucidaris tribuloides* were injected with 3H-glycine to a final activity of 25 μCi per gram wet weight. The animals were left in seawater for 45 min, 3 h, or 24 h. Jaws with teeth were subsequently dissected, fixed, and embedded by routine methods. Semithin cross sections of the dental slide were covered with Kodak AR.10 autoradiographic stripping film for 4 weeks at 18°C in the dark. The film was developed according to manufacturer's instructions. Counterstaining was with fuchsin and azur II.

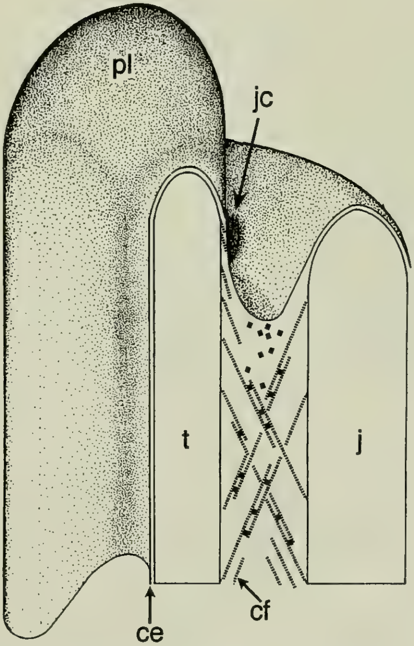
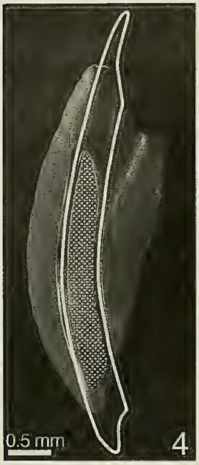


Figure 3. Schematic model of plumula and upper shaft of the tooth of *Eucidaris tribuloides* and its connection to the jaw. The right parts of tooth (t) and jaw (j) are cut away to make the tooth ligament visible. Collagen fibrils (cf) are synthesized under the coelomic epithelium (ce) that covers the plumula (pl). Small black boxes represent proteoglycans, which make cross bridges between collagen fibrils. jc: entrance of jaw canal.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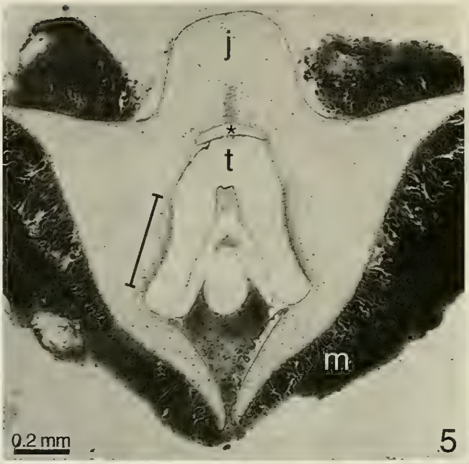
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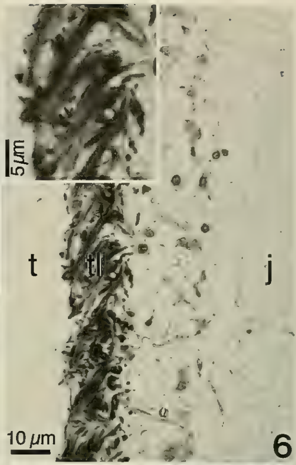
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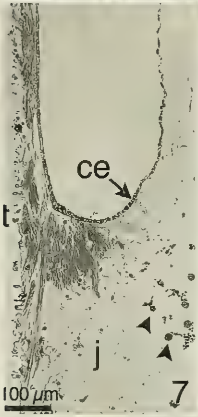
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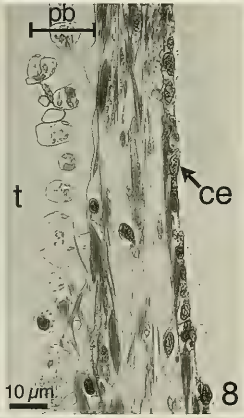
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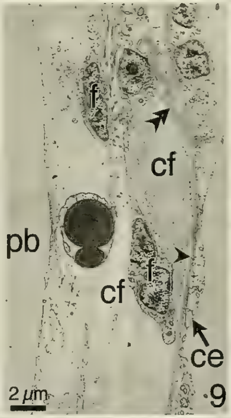
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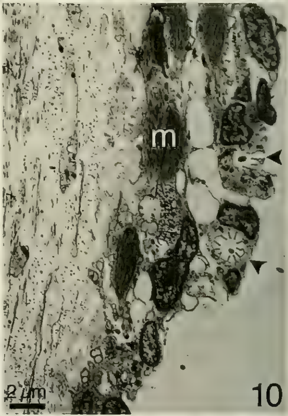
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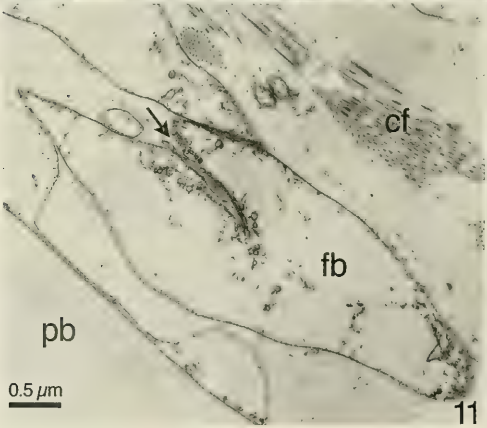
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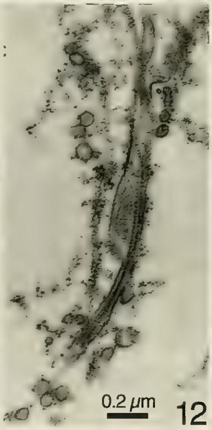
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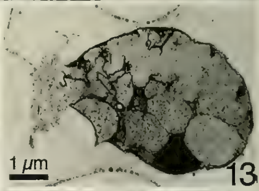
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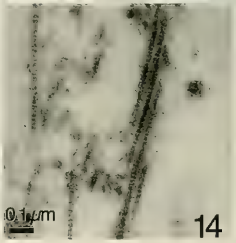
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Results

The results will refer only to the parts of the tooth that are important for the tooth ligament. For details of the inner anatomy of the tooth, see the works of Märkel (1969, 1970), Märkel and Titschack (1969), and Märkel *et al.* (1977).

Gross anatomy

The tooth of echinoids can be differentiated into three parts. The most aboral part, the plumula, is pliant and protrudes aborally from the jaw (Fig. 3). Here new tooth elements are formed. The long middle part of the tooth, the shaft, is connected to the jaw. At the oral end the tooth emerges from the jaw and forms a chewing edge that is visible in the center of the mouth. The inner surface of the jaw has two dental slides (Fig. 4). The shaft of the tooth is connected to these dental slides with a ligament that will be called tooth ligament (Fig. 5).

The tooth ligament is located in a narrow gap of 5 to 20 μm between tooth and jaw. Collagen fibril bundles of the ligament clasp around trabeculae of the skeleton of the jaw and run towards the tooth (Fig. 6). To facilitate connection to the massive tooth, the surface of the shaft region carries pillar bridges (Lovén, 1892; Märkel, 1969). Pillar bridges consist of tubercles on the surface of the tooth that bifurcate distally. The prongs of the fork elongate parallel to the longitudinal axis of the tooth and grow together. Thus long bridges with many arches are formed that range over the length of the shaft. Pillar bridges are confined to the arms of the U that the tooth forms in cross section. The beams of the U correspond exactly to the dental slides of the tooth.

The collagen fibril bundles of the tooth ligament clasp around the pillar-bridges. The fibril bundles of the tooth ligament run at acute angles to the longitudinal axis of the tooth, forming a coarse cross texture with angles of 20° to 40° and 140° to 160°, respectively. The collagen fibril bundles span distances of up to 70 μm. Cell bodies are rare in the ligament but abundant in the jaw near the dental slides.

Fine structure

Plumula region. The young tooth parts are covered by coelomic epithelium (Figs. 7, 8) consisting of a single-layered epithelium and an underlying basal lamina (Fig. 9). The epithelium consists of simple coelothel cells and muscle cells arranged lengthwise (Fig. 10). Muscle cells are especially abundant along the abaxial edges of the tooth, stretching from the soft plumula end to the entrance of the tooth into the jaw. Below this point the tooth is no longer covered by epithelium. Along the plumula, pillar bridges are built up beneath the epithelium. Collagen and microfibrils are synthesized in the mesenchymal space underlying the epithelium. In some sections, collagen fibril bundles seemed interwoven with the basal lamina of the overlying coelomic epithelium (Fig. 9). Cells are abundant among the collagen (Figs. 8, 9, 10). The typical cell in this

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Figure 4. Scanning electron micrograph of one half of a jaw. White line indicates outline of tooth when attached to the jaw. Dental slide stippled.

Figure 5. Cross section through a jaw (j) with a tooth (t). Muscles (m) insert on the outside of the jaw. Bar marks width of tooth ligament, and asterisk marks jaw canal.

Figure 6. Longitudinal section of tooth ligament (tl) connecting tooth (t) and jaw (j). Note that cell bodies are lined up in the jaw parallel to the tooth ligament; frequently sending processes towards the ligament. The inset shows a magnified view of the tooth ligament.

Figure 7. Longitudinal semithin section of the region where the tooth (t) enters the jaw (j). The upper part of the tooth and the jaw are covered by a thin coelomic epithelium (ce). Arrowheads mark some spherule cells in the jaw (see also Fig. 13).

Figure 8. Longitudinal semithin section of the plumula of the tooth (t) where collagen fibrils are synthesized under the coelomic epithelium (ce) and between the pillar bridges (pb) on the surface of the tooth.

Figure 9. Longitudinal ultrathin section of plumula. Presumed fibroblasts (f) synthesize collagen fibrils (cf). The basal lamina (arrowhead) of the coelomic epithelium (ce) seems to be interwoven with collagen fibrils in some locations (double arrowhead). pb: pillar bridge. Osmium fixation.

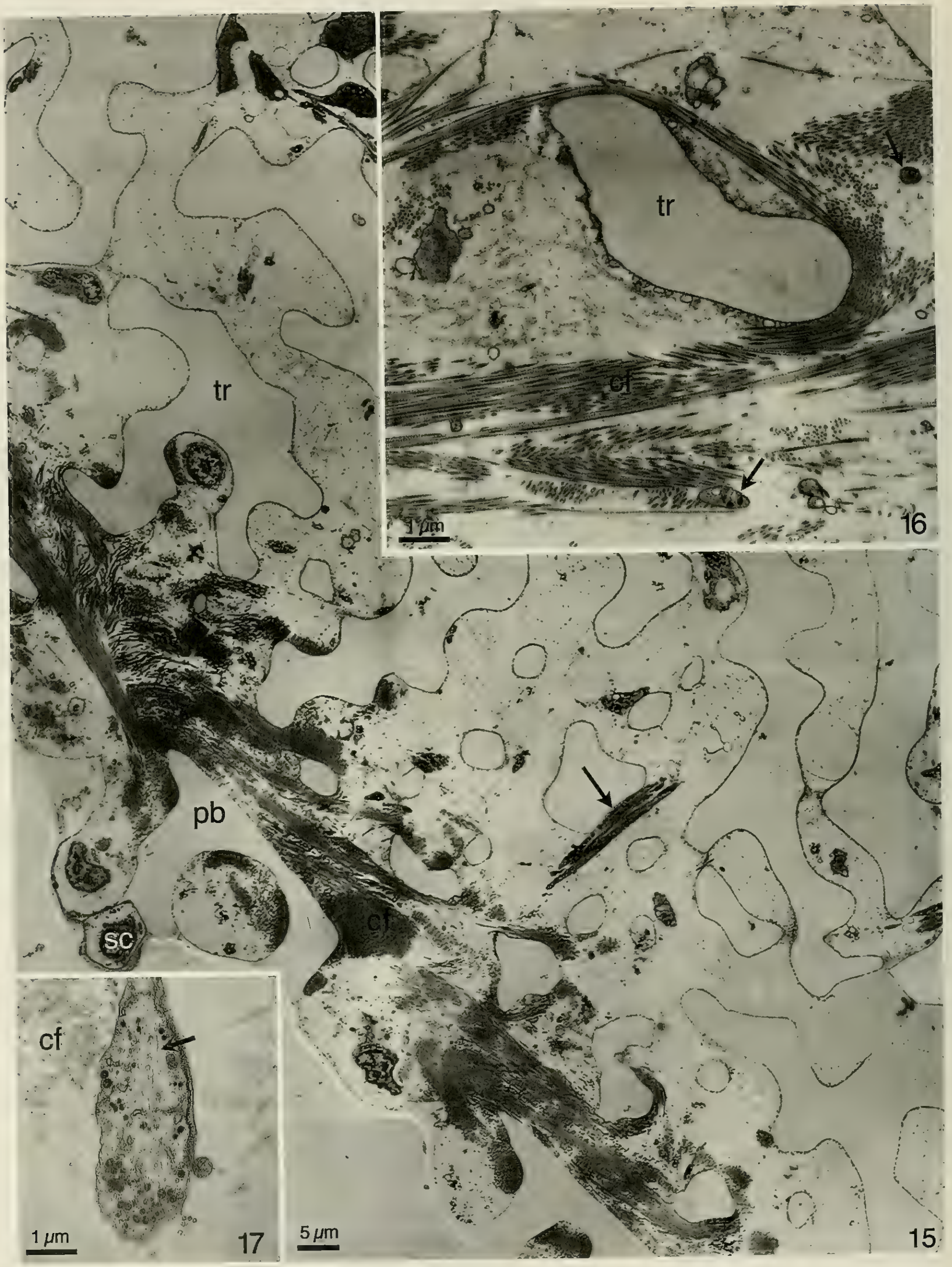
Figure 10. Ultrathin section of coelomic epithelium covering the plumula. In this section the coelomic epithelium consists of choanocyte-like cells (arrowheads) and epithelial muscle cells (m). GO fixation.

Figure 11. Ultrathin section of a presumed fibroblast (fb). Note the deep invagination (arrow) containing fibrillar material. cf: collagen fibrils, pb: pillar bridge. Osmium fixation.

Figure 12. Magnification of invagination in Figure 11. Note striped fibrils in the invagination and numerous vesicles in the cytoplasm near the invagination. Osmium fixation.

Figure 13. Ultrathin section of a spherule cell releasing granular material. The spherule was found in a location similar to that indicated by arrowheads in Figure 7. GO fixation.

Figure 14. Ultrathin section of collagen fibrils near the spherule cell of Figure 13. The fibrils are decorated with material that is most likely identical with the granular material released by the spherule cell. GO fixation.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17

region is characterized by deep invaginations that contain fine unstriped or striped fibrils (Figs. 11, 12). The fine fibrils were optimally preserved after osmium fixation, but less visible after GO fixation. In the cell, many vesicles are arranged around the invaginations. The cell also possesses mitochondria, rough endoplasmic reticulum, and a Golgi apparatus.

In the region where the young tooth parts come into contact with the jaw, the coelomic epithelium turns away from the tooth and covers the jaw instead (Figs. 3, 7). Thus the tooth and the jaw come into intimate contact, both being mesenchymal (*i.e.*, non-coelothelial) structures. In the uppermost region of the shaft, cells that resemble the spherule cells of the coelom can be found between tooth and jaw (Fig. 7) (for review of coelomocytes see Smith, 1981). These cells release fine-granular material (Fig. 13) that associates with the microfibrils and collagen fibrils (Fig. 14).

Shaft region. Under the electron microscope, cells are scarce in the tooth ligament (Fig. 15). Around the base of the pillar bridges are some scleroblasts that are not in immediate contact with the ligament. Along the whole length of the tooth ligament, almost no cell bodies are encountered amid the extracellular material. A few cell processes are the only cellular elements observed among the collagen fibril bundles (Figs. 16, 17, 18).

The extracellular material consists of collagen fibrils, microfibrils, and electron-dense granules. The collagen fibrils are arranged into thick bundles that clasp the pillar bridges of the tooth and the stereom of the jaw (Fig. 16). Collagen fibrils have diameters from 20 to 60 nm (rarely up to 100 nm) and a typical banding pattern with a periodicity of about 60 nm. Microfibrils have diameters around 10 nm and show no apparent striations under normal fixation conditions. They are arranged more-or-less parallel to the collagen bundles and are sometimes connected with them (Fig. 19). While aggregating to loose bundles, microfibrils are not strictly parallel to each other. The distance between them varies, and sometimes they seem to be connected to each other. The dark granules are irregularly shaped and have diameters from 10 to 30 nm. They are often arranged in groups. They can be found next to microfibrils or collagen fibrils, but also free in the extracellular space (Fig. 19).

Cell bodies in the jaw are abundant at a distance of around 20 μm from the insertion of the tooth ligament (Fig. 6). They belong to two cell types that we call dense vesicle cells (dv cells) and supporting cells (Figs. 20, 38). These two cell types are always found in close association. The cell body of a dv cell contains the irregularly shaped nucleus and other organelles (Fig. 20). The Golgi apparatus near the nucleus produces dense vesicles with a diameter of up to 300 nm. These dense vesicles are located in the cell body and in long cell processes that extend among the collagen fibril bundles of the tooth ligament (Fig. 16). The cell processes contain microtubules (Fig. 17).

The supporting cells have an elongated shape and are characterized by an indentation containing a short cilium (Figs. 22, 23). The indentation is separated from the extracellular space by cytoplasmic rods. The rods are interconnected by a delicate membrane (Fig. 25). Coated pits and coated vesicles can be frequently observed in the region of the indentation. Rough endoplasmic reticulum and Golgi apparatus of the supporting cells are often distended and sometimes filled with granular material (Fig. 24).

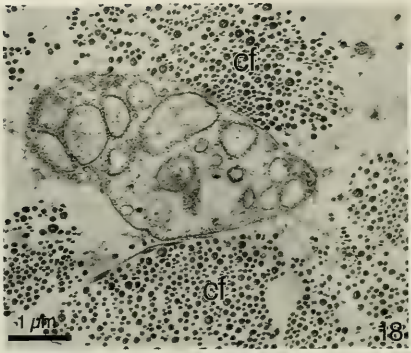
A unique feature of the supporting cells is a cord of parallel fibrils that can be several micrometers long. Under normal glutaraldehyde osmium fixation, the cord has an indistinct striation of 300 nm (Fig. 20). If fixation is only with osmium, the striation is more distinct, but of different periodicity (Fig. 21). A bright region of around 225 nm is separated by a row of dark globules from a dark region about 150 nm long. Another row of dark globules is followed by another bright region. A double row of dark globules finishes one period, which thus has a total length of 600 nm, twice that observed with glutaraldehyde osmium fixation. Between the collagen fibrils we sometimes observed cell profiles that are probably processes of the supporting cells (Fig. 18).

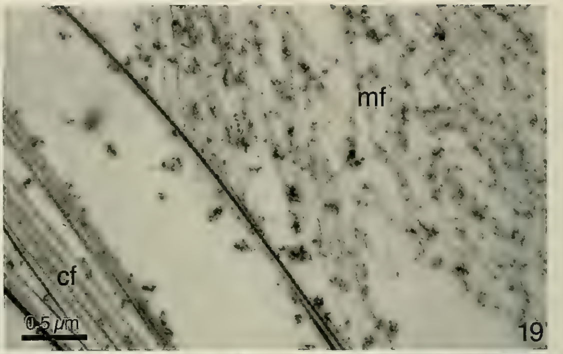
Chewing edge. When the tooth leaves the jaw orally during growth, the pillar bridges on its surface vanish (Märkel, 1969; Märkel and Titschack, 1969) and the ligament is dissolved and disappears (Fig. 26). In this region of degeneration a number of cells can be observed in the ligament (Fig. 27). In ultrathin sections we found phagocyte-like cells that obviously take up collagen (Fig. 28). These cells have dense cytoplasm and an elongated shape. They are arranged along the collagen fibril bundles of the

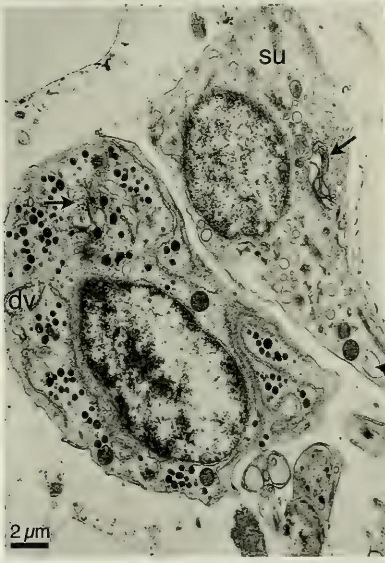
Figure 15. Ultrathin section of tooth ligament. Collagen fibrils (cf) clasp the trabeculae (tr) of the jaw and the pillar bridges of the tooth (pb). It is not clear, however, whether one single fibril attaches to both tooth and jaw. Cells are rare among the collagen. Some sclerocytes (sc) can be seen at the base of the pillar bridges. They have no contact to the ligament. Arrow points to processes of dense vesicle cells running through the jaw towards the ligament. GO fixation.

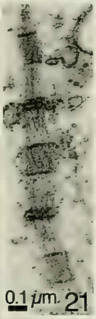
Figure 16. Ultrathin section of collagen fibrils (cf) clasping a trabeculum (tr) of the jaw. Arrows mark processes of dense vesicle cells among the collagen. GO fixation.

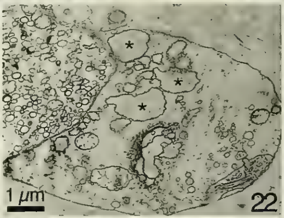
Figure 17. Ultrathin section of a process of a dense vesicle cell located next to collagen fibrils (cf) of the ligament. The process is stabilized by microtubules (arrow) along its longitudinal axis. GO fixation.

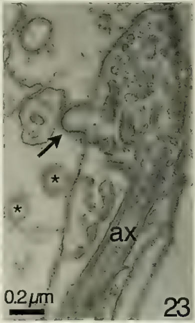


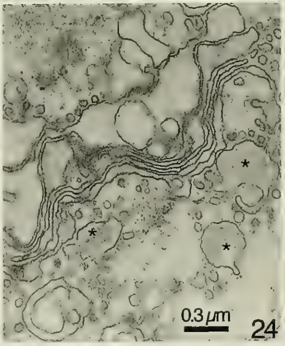












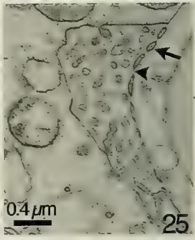


Figure 18. Ultrathin section of a cell process among collagen fibrils (cf) of tooth ligament. The process contains numerous vacuoles that are similar to vacuoles of supporting cells found in the jaw (Fig. 22). Osmium fixation.

Figure 19. Ultrathin section of tooth ligament. The ligament consists of collagen fibrils (cf) and microfibrils (mf), both decorated with dark granules. GO fixation.

Figure 20. Ultrathin section of dense vesicle cell (dv) and supporting cell (su) in the jaw (see also Fig. 38). Arrows point to Golgi complexes. The supporting cell has a fibrillar chord (arrowhead) along a cell process. GO fixation.

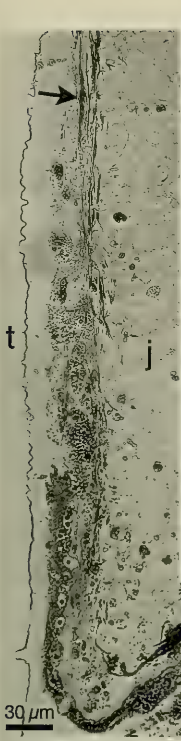
Figure 21. Ultrathin section of the fibrillar chord of a supporting cell with a distinct structure after osmium fixation.

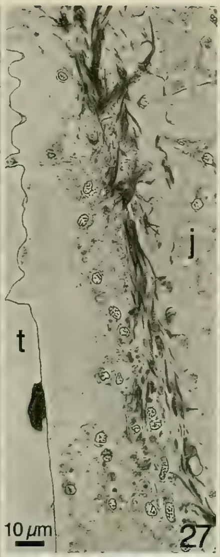
Figure 22. Ultrathin section of a supporting cell containing numerous vacuoles (asterisks) with fine granular contents. Osmium fixation.

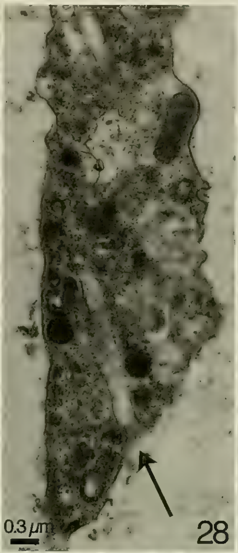
Figure 23. Magnification of indentation of a supporting cell in Figure 22. The indentation contains some short microvilli-like processes and a long process containing an axoneme-like structure (ax). Arrow marks a coated pit and asterisks mark coated vesicles. Osmium fixation.

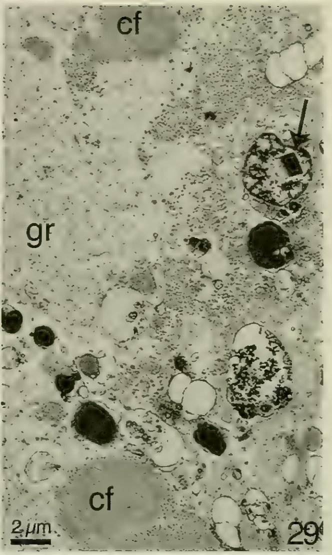
Figure 24. Ultrathin section of a Golgi apparatus of a supporting cell. Asterisks mark vacuoles containing fine granular material. Osmium fixation.

Figure 25. Ultrathin section of indentation of a supporting cell. This section shows the cytoplasmic rods (arrow) and the thin interconnecting membrane (arrowhead) separating the indentation from the outside. Osmium fixation.





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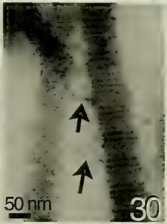

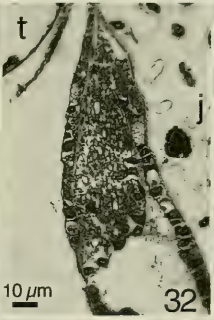

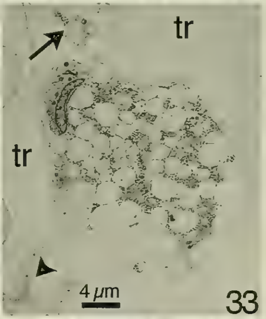

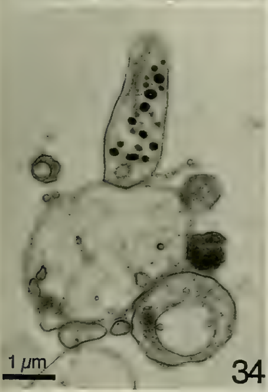

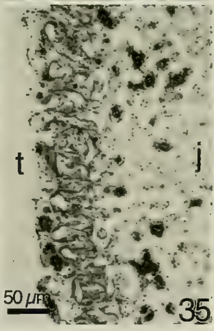

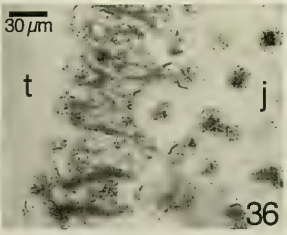

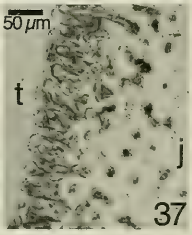

Figure 26. Semithin longitudinal section of tooth (t) and jaw (j) in the region where the tooth protrudes from the jaw (at the bottom of the photograph). The pillar bridges on the surface of the tooth have vanished. The ligament can be seen at the top of the micrograph (arrow), but gradually degrades towards the bottom, where a number of cells and extracellular material of indistinct structure fill the space between tooth and jaw.

Figure 27. Semithin longitudinal section through the vanishing tooth ligament. The space between tooth (t) and jaw (j) is crowded with cells that replace the ligament.

Figure 28. Ultrathin section of a presumed phagocyte in the degrading tooth ligament. Arrow points to an invagination that contains collagen-like fibrils. GO fixation.

tooth ligament. Other cells resemble late phagocytes—*i.e.,* phagocytes that have taken up waste material. They have a crystalloid in their nucleus and contain roundish aggregations of electron-dense material (Fig. 29).

We examined the extracellular matrix of the region of degeneration and found that collagen bundles and microfibrils are replaced by granular structures (Fig. 29). The collagen fibrils have diameters of up to 200 nm, and a statistical survey of 951 fibrils of the tooth ligament and 1023 fibrils of the oral region (Fig. 39) shows that the collagen fibrils swell in the oral region. The results were the same in samples treated by three different fixations, so fixation artifacts can be excluded.

Histochemistry

Light microscopy. Several standard methods allow specific staining of glycosaminoglycans. With these methods, we compared different parts of the echinoid body (Table I). These results show that acid glycosaminoglycans are comparatively rare in muscles, in stroma of tooth, and in stroma of jaw, but can be detected in the tooth ligament and in the pyramidal suture of jaws, as shown by strong staining with alcian blue at pH 2.5 and with colloidal iron. The tooth ligament stained with alcian blue at pH 0.2, indicating the presence of sulfated glycosaminoglycans. Alcian blue plus MgCl in increasing critical electrolyte concentration detects glycosaminoglycans of increasing grades of sulfatation (Scott and Dorling, 1965). Clearly visible staining even at a magnesium concentration of 0.9 *M* indicates that the glycosaminoglycans of the tooth ligament are highly sulfated.

Electron microscopy. Tannin in a fixation solution was used for visualization of hyaluronic acid (Singley and Solursh, 1980). In the case of tooth ligament, tannin revealed fine fibrils that interconnect the collagen (Fig. 30), and it made collagen banding crisp. Safranin O in the fixation solution binds specifically to proteoglycans (Shepard and Mitchell, 1976). Electron micrographs of samples fixed with this schedule showed the collagen fibrils decorated with dark granules that were regularly arranged with the same periodicity as the collagen banding pattern (Fig. 31).

Innervation of jaw and tooth ligament

The nervous system of echinoids is radially organized. A circumoral nerve ring encircles the pharynx and sends off five radial nerve cords (Fig. 40). In *Eucidaris tribuloides,* we found that the radial nerve sends off two additional nerve cords, that we name jaw nerves, in each interradius. They run along the inside of the jaw near each dental slide (Figs. 2, 32). Four smaller nerve strands branch off from each jaw nerve. Each branch goes through a pit on the inner surface of the jaw and runs abradially through the stereom of the jaw. These branches probably innervate the protractor and postural muscles that insert on the outside of the jaw (Fig. 40).

In some places the branches run near the tooth ligament (Fig. 33). In these regions, dv cells are in close contact with the axons (Fig. 34). Electron microscopically, the nerve consists of different kinds of axons. One type has a diameter of about 2 μm and appears electron-lucent with transmission electron microscopy. The second type is filled with granular material of medium electron density and has a diameter of 0.5 to 1 μm. It often contains dark vesicles and sometimes clear vesicles. The third type of axon has a diameter of about 0.1 μm and appears electron-dense.

Autoradiography

Forty-five minutes after injection of ^{3}H-glycin, cells in the jaw along the tooth ligament and cells at the base of

Figure 29. Ultrathin section of degrading tooth ligament. Some bundles of collagen fibrils can still be found (cf), but most of the extracellular space is filled with granular material (gr). Phagocytes are abundant and characterized by a crystalloid (arrow) in their nucleus. GO fixation.

Figure 30. Ultrathin section of collagen fibrils after fixation in glutaraldehyde solution containing 1% tannin. Thin fibrils interconnect the collagen fibrils (arrows).

Figure 31. Ultrathin section of a collagen fibril after fixation in glutaraldehyde solution containing 0.1% Safranin. Dark spots cover the surface of the collagen fibrils. The distance between the spots corresponds to the banding pattern of collagen.

Figure 32. Semithin cross section of jaw nerve. The jaw nerve is located near tooth (t) and jaw (j). See also Figure 2.

Figure 33. Ultrathin cross section of a branch of the jaw nerve running between the trabeculae (tr) of the jaw near the tooth ligament. A dense vesicle cell is in close contact with the nerve (arrow, see also Fig. 34). Arrowhead points to some collagen fibrils of the tooth ligament. GO fixation.

Figure 34. Magnified view of Figure 33. Dense vesicle cell in contact with presumed axons. GO fixation.

Figure 35. Autoradiograph of semithin section of tooth ligament. Fixation was 45 min after application of ^{3}H-glycine. Cells at the base of the pillar bridges of the tooth (t) and in the jaw (j) are strongly marked.

Figure 36. Autoradiograph of semithin section of tooth ligament. Fixation was 45 min after application of ^{3}H-glycine. Markers in the tooth ligament are rarely located over collagen fibril bundles.

Figure 37. Autoradiograph of semithin section of tooth ligament. Fixation was 3 h after application of ^{3}H-glycine. Markers can only be found over cells in the jaw (j). t: tooth.

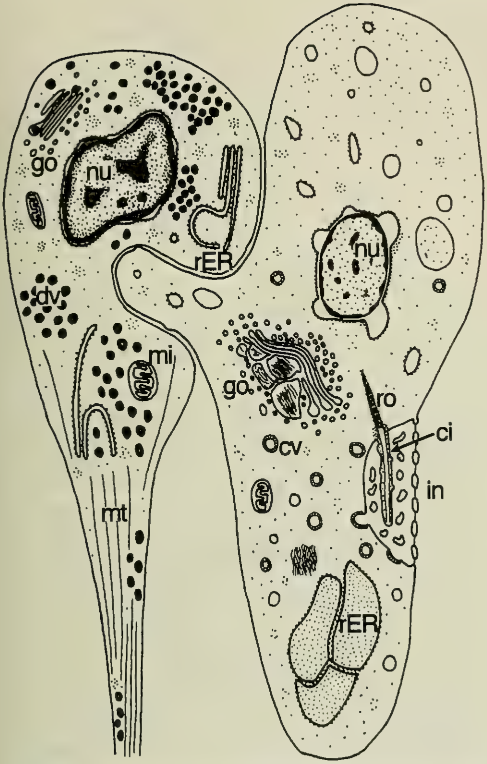

Figure 38. Schema of dense vesicle and supporting cell constructed from various sections. ci: cilium, cv: coated vesicle, dv: dense vesicle, go: Golgi apparatus, in: indentation, mi: mitochondrion, mt: microtubules, nu: nucleus, rER: rough endoplasmic reticulum, ro: rootlet.

the pillar bridges of the tooth were marked intensely (Fig. 35). Collagen fibril bundles were marked rarely, whereas the extracellular matrix between the fibril bundles was marked in many places (Fig. 36). Three hours after injection, cells along the tooth ligament, cells between the pillar bridges, and the extracellular matrix were less marked than after 45 min (Fig. 37). However, cells in the jaw at a distance from the tooth ligament were marked. Twenty-four hours after injection, markers were observed only rarely.

Discussion

The continuously growing tooth of the sea urchin is functionally comparable to the continuously growing incisor of the rodent. Both teeth have to be connected firmly to the jaw to withstand stresses during feeding, and at the same time the ligament has to allow slow shift of the tooth along the jaw. The periodontal ligament of the mouse consists of layers of collagenous tissue intercalated by layers of fibroblasts that make up about 50% of the ligament area (Ten Cate *et al.,* 1976). The fibroblasts are capable of simultaneously synthesizing and degrading collagen. The peridodontal ligament is remodeled continuously, allowing slow movement of the tooth. The tooth ligament of sea urchins contains almost no cells. Furthermore, our autoradiographic data show that the turnover rate of collagen is slow, indicating a different mechanism than in the periodontal ligament of the mouse.

Extracellular matrix

The tooth ligament contains microfibrils decorated with dark granules. During fixation, proteoglycans often collapse to dark granules (Hascall, 1980; Thyberg *et al.,* 1973). Collagen is normally associated with proteoglycans that consist of a protein "backbone" and glycosaminoglycan side chains. The results of our histochemical tests with alcian blue and colloidal iron show that the extracellular matrix of the tooth ligament contains proteoglycans that carry side chains of highly sulfated acid glycosaminoglycan. Proteoglycans can be associated with hyaluronic acid (Hascall, 1974). After a fixation that includes 1% tannin, we observed thin filaments interconnecting the collagen fibrils. It seems possible that these filaments are part of the hyaluronic backbone of proteoglycan ag-

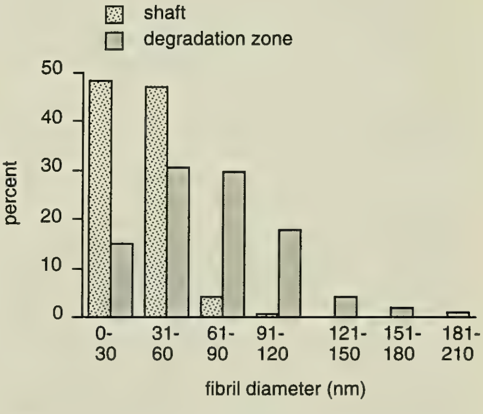

Figure 39. Distribution of collagen fibril diameter in two regions of the tooth ligament. In the region of the shaft, the ligament connects the tooth to the jaw. In the degradation zone, where the tooth protrudes from the jaw, collagen is degraded. Horizontal axis is fibril diameter in 30-nm intervals; vertical axis is percentage of each interval.

Table I

Light microscopic histochemistry of various jaw-related tissues of Eudicaris tribuloides

Stain*	pH	Tissue†				
		Tooth	Jaw	Tooth ligament	Jaw suture	Interpyramidal muscle
Colloidal iron reaction	NA	+	+	+++	+++	0
1% AB	0.2	+	+	++	+	0
1% AB	2.5	++	++	+++	+++	0
1% AB and 0.05 *M* $MgCl_2$	2.5	+	+	+++	++	0
1% AB and 0.3 *M* $MgCl_2$	2.5	+	+	+++	++	0
1% AB and 0.5 *M* $MgCl_2$	2.5	+	+	++	+	0
1% AB and 0.7 *M* $MgCl_2$	2.5	+	+	++	+	0
1% AB and 0.9 *M* $MgCl_2$	2.5	0	0	+	+	0

* AB = alcian blue.

† 0 indicates no staining; + indicates weak staining; ++ indicates intense staining; +++ indicates very intense staining.

gregates. Under normal fixation conditions, such filaments were not visible. Normal fixation, however, causes proteoglycans to collapse (Kagami *et al.,* 1990). The dark granules of the extracellular matrix might be remnants of an extensive three-dimensional network of proteoglycan aggregates.

Microfibrils can consist, at least partly, of fibrillin (Sakai *et al.,* 1991; Shuttleworth *et al.,* 1992) or of type VI collagen (Waggett *et al.,* 1993). Their function has not been definitely established, but they are often found in elastic tissues (Gerli *et al.,* 1990; Keene *et al.,* 1991). In the tooth ligament they might function as shock absorbers when the tooth scratches on uneven rock surfaces.

In summary, the ultrastructure of the tooth ligament of sea urchins is different from that of the mammalian periodontal ligament. An extracellular matrix of collagen fibrils, microfibrils, and proteoglycans is typical for echinoderm catch connective tissues (Wilkie, 1984) that can change their stiffness under nervous control.

The cells of the tooth ligament

The cells in the plumula region resemble fibroblasts. They are found in the collagen-filled space below the coelomic epithelium. They possess rough endoplasmic reticulum and a Golgi apparatus, indicating their secretory activity. Cytoplasmic vesicles near invaginations that contain thin fibrils are also typical for fibroblasts. We think that these cells synthesize collagen that becomes part of the tooth ligament during the slow movement of the tooth. The morula cells in the jaw where the tooth comes into contact with the jaw resemble the morula cells that deliver components of the extracellular matrix during wound repair (Smith, 1981). They probably secrete components of the extracellular matrix that interconnect the collagen fibrils of the tooth ligament.

The main cell types of the shaft region are the dense vesicle cells (dv cells) and the supporting cells. Dv cells closely resemble the juxtaligamental cells that are typical for echinoderm catch connective tissue (Motokawa, 1984; Wilkie, 1984). They have been also called neurosecretory cells because of their resemblance to vertebrate neurosecretory cells, although their neurosecretory character has never been established. In the tooth ligament they connect to the nervous system, but their function remains obscure. The unique structure of the supporting cells indicates that they might be involved in both the uptake and synthesis of some material. The coated pits could be responsible for uptake, and Golgi apparatus and rough endoplasmic reticulum for synthesis. The cytoplasmic bridges and the interconnecting membrane covering the indentation resemble podocytes described for the echinoid axial organ (Welsch and Rehkämper, 1987). Unlike the podocytes of the axial organ, these supporting cells are not associated with a basal lamina, but there is little doubt that they are involved in the filtering of extracellular fluid. Märkel *et al.* (1992) gave the name filter cells to similar cells from the sphaeridia of *Asthenosoma varium.*

The vacuoles of supporting cells resemble calcium-containing vacuoles of cells found in the holothurian dermis (Matsuno and Motokawa, 1992). They are thought to be responsible for the catch mechanism (reversible stiffening of the dermis) by release and re-uptake of calcium. It can be speculated that the supporting cells receive signals from the closely associated dv cells that are in connection with the nervous system. Their processes would release or take up calcium, causing stiffness changes of the tooth ligament. The podocyte-like structure might serve for selective pinocytosis of calcium.

The cells of the chewing edge where the tooth leaves the jaw resemble phagocytes filled with electron-dense material typical for cells involved with waste removal (Smith, 1981). Variable diameter of collagen fibrils found in this region and their breakdown into a granular deposit

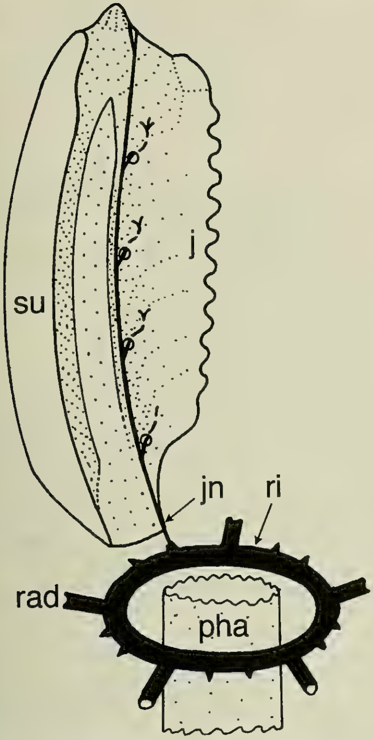

Figure 40. Schema of innervation of jaw of *Eucidaris tribuloides.* A half jaw (j) is shown from the inside with the tooth removed. The circumoral nerve ring (ri) encircles the pharynx (pha) and gives rise to five radial nerves (rad) and 10 jaw nerves (jn), one for each half jaw. Branches of each jaw nerve penetrate through pits into the stereom of the jaw. These branches sometimes run near the tooth ligament. su: suture where two half jaws are connected.

are typical for collagenolysis, as, for example, in the human uterine cervix when giving birth (Junqueira *et al.,* 1980).

Innervation

Innervation of the lantern of Aristotle was investigated only by Cobb and Laverack (1966), who described two nerves running on the outside and the inside of the jaw. They based their findings on staining with methylene blue, making the distinction of nervous tissue from connective tissue difficult. For better identification of nervous tissue, we used an osmic acid fixation and examined whole preparations and serial semithin sections. Our findings show that, at least in *Eucidaris tribuloides,* there is no nerve on the outside of the jaw. We also examined three other species (*Centrostephanus longispinus, Psammechinus miliaris, Arbacia lixula;* data not shown) and found nerves only on the inside of the jaw. As our data show, the jaw nerves are connected to the main nervous system of the sea urchin on one hand and to the dv cells of the tooth ligament on the other hand. We can conclude that the tooth ligament is connected to the sea urchin's main nervous system.

Model

We propose the following model of how the tooth ligament functions.

During growth, the tooth slowly slides along the jaw. In the plumula region, collagen is synthesized between the pillar bridges on the surface of the tooth and the overlying coelomic epithelium. The growing tooth transports the collagen orally. When it comes into contact with the jaw, the collagen connects the tooth with the jaw. This is facilitated by cross-bridges between collagen on the tooth side (which is transported) and collagen on the jaw side (which is static). Cross-bridges consist of extracellular matrix elements, mainly proteoglycans and microfibrils.

In response to a nervous impulse, dv cells cause supporting cells to release or take up calcium. As in the mechanism proposed for the catch connective tissues of other echinoderms, changes in calcium concentration induce changes in the stiffness of the ligament. When the tooth is subject to external forces, the ligament is stiff and connects the tooth firmly to the jaw. When the tooth is not loaded, the ligament is soft and allows slow shift of the tooth along the dental slide. Evidence for this mechanism and a detailed explanation are given in the following paper (Birenheide *et al.,* 1996). The collagen of the tooth side is slowly transported orally, sliding along the static collagen of the tooth side. It finally reaches the region where the tooth leaves the jaw. Here the collagen is depolymerized to granular material and is taken up by phagocytes.

Acknowledgments

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To be Stiff or to be Soft—the Dilemma of the Echinoid Tooth Ligament. II. Mechanical Properties

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Abstract. The teeth of sea urchins are connected to jaws by means of ligaments. Their sliding along the jaw during continuous growth requires a pliant ligament, whereas scraping on rocks for feeding requires a stiff ligament for firm support. We investigated the mechanical properties of the tooth ligament of *Diadema setosum* to clarify how sea urchins solve this dilemma. In creep tests a load of 30 g caused a shift of the tooth that continued until the tooth was pulled out of the jaw. The creep curve had three phases: an initial phase of high creep rate, a long phase of constant creep rate, and a final phase of accelerating creep rate. The ligaments had a shear viscosity of about 550 MPa • s. Viscosity increased reversibly after stimulation with seawater containing a high concentration of potassium ions or acetylcholine. Frozen and rethawed ligaments did not show an increase of viscosity after stimulation. The data indicate that sea urchins can change the stiffness of their tooth ligaments through nervous control. We suggest that the tooth ligament is a catch connective tissue.

Introduction

Collagenous ligaments connect the teeth of sea urchins to their jaws. These ligaments are faced with contradictory demands: they have to be stiff to hold the teeth firmly when the sea urchin scrapes rocks and hard surfaces during feeding, but they have to be soft enough to allow shift of the teeth towards the outside as new tooth material is added to the inner (aboral) growth zone to compensate for wear. A similar dilemma is found in rodent teeth. Our morphological studies showed, however, that the collagen remodeling mechanism that solves the problem in rodents (Beertsen and Everts, 1977) is not likely to be the answer for echinoids (Birenheide and Motokawa, 1996). Instead, a possible solution lies in the use of catch connective tissue. This is a connective tissue peculiar to echinoderms and unique in its ability to change its mechanical properties under nervous control (Motokawa, 1984a; Wilkie, 1984). In this study we investigated the mechanical properties of echinoid tooth ligament and their possible nervous control. We present data supporting the idea that sea urchins employ catch connective tissue to solve their dilemma. This is the first physiological study ever conducted on sea urchin tooth ligaments.

Materials and Methods

Specimens of the sea urchin *Diadema setosum* Leske were obtained near Misaki Marine Biological Station (University of Tokyo), Sagami Bay, Japan. They were kept in aquaria with circulating artificial seawater for no more than 6 months. Lanterns were dissected and divided into five preparations, each consisting of a jaw with a tooth. Experiments were conducted in artificial seawater (ASW), ASW with elevated potassium concentration (KASW), and ASW containing acetylcholine (ACh). The composition of ASW was NaCl 433.7 mM; KCl 10.0 mM; $CaCl_2$ 10.1 mM; $MgCl_2$ 52.5 mM; $NaHCO_3$ 2.5 mM (pH 8.0). The composition of KASW was NaCl 343.7 mM; KCl 100.0 mM; $CaCl_2$ 10.1 mM; $MgCl_2$ 52.5 mM; $NaHCO_3$ 2.5 mM (pH 8.0). Acetylcholine (ACh) was added to ASW to make a final concentration of 10^{-3} M or 10^{-4} M. All experiments were performed at room temperature. The chemicals were washed out by thorough rinse with several changes of ASW. The mechanical properties of fresh ligaments were compared with those of frozen control ligaments. Frozen ligaments had been kept for more than 1 week at −20°C, then rethawed in ASW for several hours at room temperature. For mechanical testing, a jaw was

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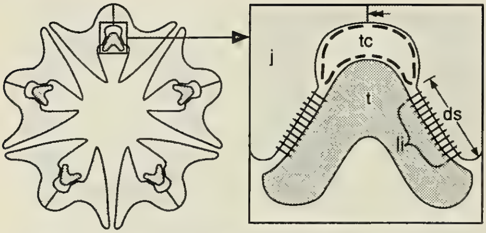

Figure 1. Schematic cross section of Aristotle's lantern of *Diadema setosum* (left) and magnified view of jaw (j) with dental slides (ds), tooth (t), and tooth ligament (li). Note the extensions of the tooth coelom (tc) along the sides of the tooth. The jaw consists of two pieces connected along a suture (double arrow).

glued with cyanoacrylate glue to a platform in a trough containing ASW.

A load of 30 g was applied to the tooth by means of a lever. In pull tests the tooth was pulled in the same direction as it naturally shifts during growth. In these tests the lever was connected to the tooth with a silver wire. In push tests the tooth was pushed in the opposite direction as in pull tests, and the lever was connected with a plastic holder. Displacement of the tooth was detected by a linear eddy current sensor (502-F, EMIC Corp., Tokyo, Japan) and recorded with a microcomputer.

Teeth that had been pulled out of the jaw in mechanical tests, and the corresponding jaws, were examined by scanning electron microscopy. Specimens were fixed in 3.5% glutaraldehyde in 0.1 *M* cacodylate buffer, postfixed with 1% osmium tetroxide in the same buffer, and dehydrated in an acetone series. After an intermediate step of amyl acetate, they were dried in a critical point drying apparatus and mounted on holders. The specimens were then coated with gold and observed in a JEOL JSM-T220 scanning electron microscope.

Results

Anatomy

The tooth ligament of *Diadema setosum* is morphologically similar to the ligament of *Eucidaris tribuloides* described in the preceding paper (Birenheide and Motokawa, 1996). The main difference is in the size of the tooth ligament. In *D. setosum* the tooth ligament is shorter in relation to the whole length of the jaw. The tooth coelom on the abradial side extends along both sides of the U-shaped tooth and narrows the tooth ligament (Fig. 1). The length of the dental slide varied with size of the lantern, although we could not establish a clear allometric relationship. Average length was 11.7 mm (SD = 1.83, n = 104). Thickness and width of tooth ligaments were measured in semithin sections of different samples from average-sized lanterns. Average thickness was 24.8 μm (SD = 11.1, n = 13) with the adradial parts being thinner than the abradial parts. Width showed a rather constant value of 600 μm.

As in *E. tribuloides,* the collagen fibril bundles made up a rough crosstexture. The angles between the fibril bundles and the longitudinal axis of the tooth were 20° to 40°. If we consider a minimal angle of 20° and a tooth ligament thickness of 25 μm, the maximal length of a collagen fibril bundle would be no more than about 150 μm.

Creep tests in artificial seawater

When a load was applied in pull tests, the tooth slowly shifted until it was pulled out of the jaw. The time span until the tooth was extracted varied between 25 min and 67 h. In all cases the ligament finally failed and the tooth was pulled out. The surface of both tooth and jaw after failure carried a great number of collagen fibril bundles clasping either to the pillar bridges of the tooth or to the trabeculae of the jaw (Fig. 2). Most fibrils were oriented parallel to the longitudinal axis of the tooth. At failure of the ligament, the tooth had shifted for 550 μm (SD 230, n = 6), corresponding to about 4.7% of the length of the dental slide.

The creep curves were characterized by three phases (Fig. 3A): a short initial phase with a high creep rate, a second phase with a slow, constant creep rate, and a third phase with a creep rate that gradually increased until the

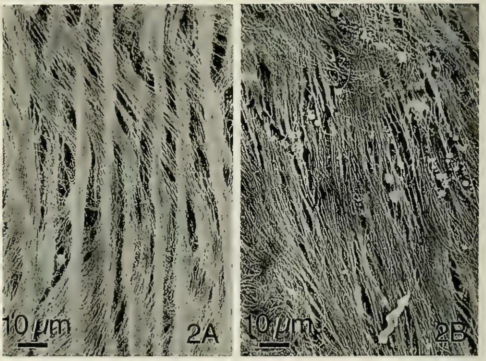

Figure 2. Scanning electron micrographs of the surfaces of tooth (2A) and of corresponding dental slide of the jaw (2B) of *Diadema setosum* after the tooth was pulled out of the jaw during the experiment. The micrographs are oriented with the tip of the tooth to the bottom. Both surfaces are covered by a great many fibrils, probably collagenous. The fibrils conceal the underlying skeleton almost completely.

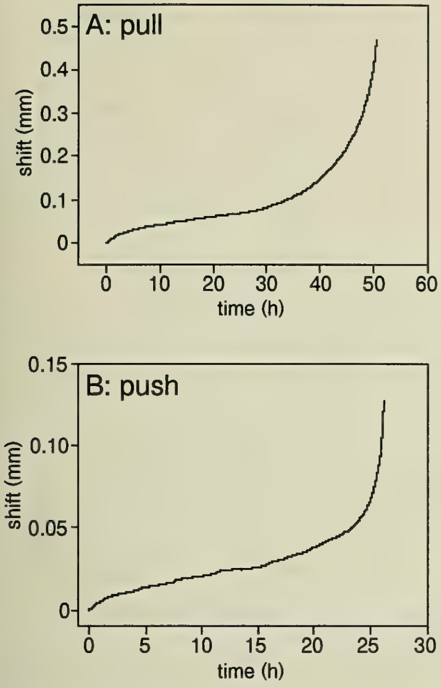

Figure 3. Creep curves from loading test of tooth. Curves from pull tests (A) and push tests (B) have similar shape. Upper right end of each curve indicates the point at which the tooth was pulled out of the ligament.

ligament failed. The average creep rate in the constant second phase was 6.8 μm/h (SD 6.1, $n = 36$).

Relative viscosity was defined as the reciprocal of creep rate, with the value just before application of a test solution taken as unity.

Controls were push tests and tests with frozen jaws. In push tests we observed no significant differences from pull tests. The creep curves were similar (Fig. 3B). Experimental time until the tooth was pushed out was 17 to 97 h. The tooth had shifted 460 μm (SD 170, $n = 6$) until failure. The creep rates during the second phase were of the same order as in pull tests.

Freezing and rethawing of the preparations did not change the shape of the creep curves or the creep rates. Shift until failure in pull tests was 820 μm (SD 410, $n = 4$) and in push tests was 430 μm (SD 160, $n = 7$).

Responses to stimuli

Acetylcholine (ACh) caused a decrease in creep rate (Fig. 4A); *i.e.*, the ligament stiffened. In 17 out of 23 tests, relative viscosity doubled in 15 min (see Fig. 6). The effect was reversible: the creep rate recovered in about 1 h after washing with artificial seawater. Treatment with artificial seawater containing 100 mM K^+ (KASW) also stiffened the ligament (Fig. 5A). Relative viscosity doubled in 15 minutes in 6 out of 8 tests. This effect, too, was reversible.

Push tests showed no difference in response to stimuli. ACh caused stiffening of the ligament. In two of three cases, relative viscosity increased more than 10-fold (Fig. 6). KASW caused increase of relative viscosity in all three cases—in two of them more than sixfold (Fig. 6).

In extreme cases stimulation stopped movement of the tooth; *i.e.*, the ligament did not creep under the load of 30 g. This reaction was observed in four cases after application of ACh in pull tests and in two cases after application of KASW, once in pull tests and once in push tests.

In frozen ligaments, ACh had no effect on the creep rate in the first 15 min after stimulation (Fig. 4B). Creep

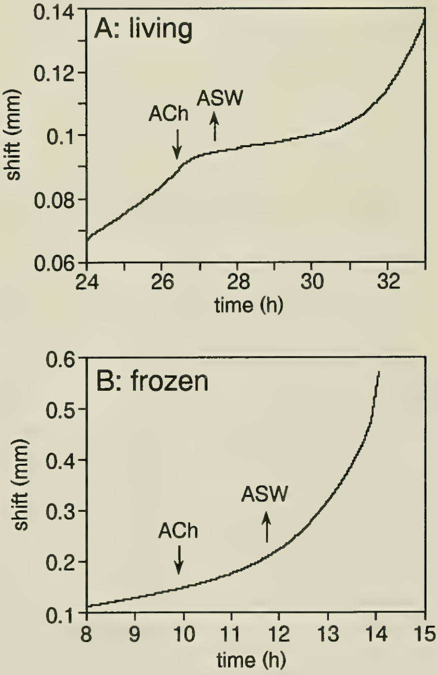

Figure 4. Influence of acetylcholine (ACh) in pull tests. Application of 10^{-3} M ACh (downward arrow) decreased creep rate of living ligaments (A), but had no effect on frozen and rethawed ligaments (B). Upward arrow denotes wash with ASW. Only a portion of the creep curve is shown, as indicated by the time scale.

rate either did not change or increased slightly (Fig. 6). The increase is not due to ACh because in all experiments ACh was applied in the third phase (accelerating creep rate) in ASW and because the increase was not reversible when ACh was washed out by ASW. Although the effect was not obvious in 15 min, KASW caused an increase in creep rate (Fig. 5B). Creep rate doubled; *i.e.*, relative viscosity halved within 1 h after application of KASW. In some cases the viscosity dropped considerably, even in the first 15 min after the stimulus (Fig. 6). The creep rate recovered after washing with artificial seawater of normal potassium concentration. This reversible softening of the ligament was observed in almost all frozen preparations regardless of whether pull tests or push tests were used.

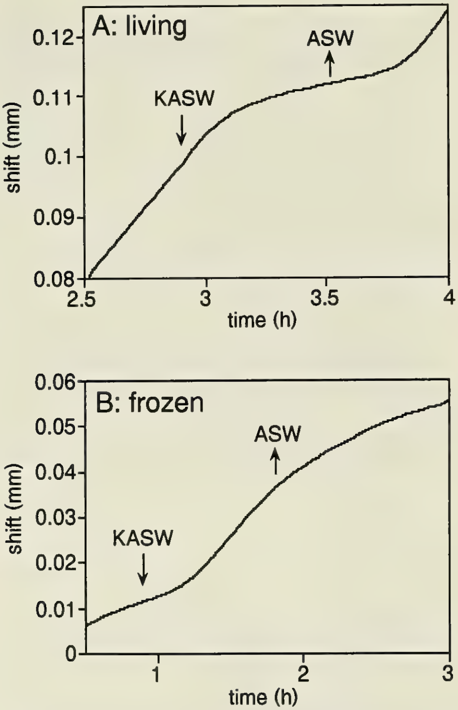

Figure 5. Effect of artificial seawater containing 100 m*M* potassium ions (KASW) on the creep curve in pull tests. KASW decreased the creep rate of living ligaments (A), but had the opposite effect on frozen and rethawed ligaments (B). KASW was applied in the third phase of the creep curve in A and in the second phase in B, because a decrease can be demonstrated more clearly in the third phase, which has a high creep rate (A), whereas an increase can be demonstrated more clearly in the second phase, which has a low creep rate (B).

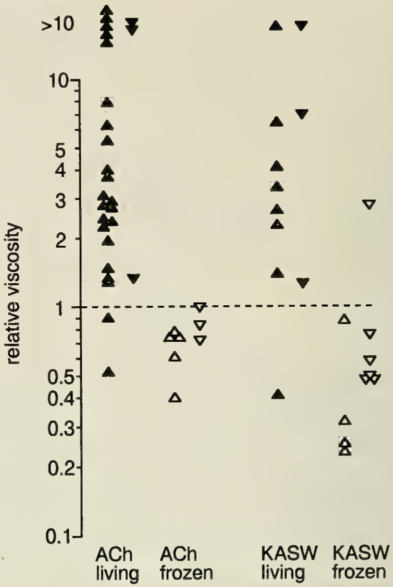

Figure 6. Relative viscosity of the tooth ligament 15 min after stimulation. Upward arrowheads refer to pull tests, downward arrowheads to push tests. ACh = acetylcholine; KASW = artificial seawater with 100 m*M* potassium ions. Relative viscosity at unity indicates no change. Most stimulation was applied in the final accelerating phase of the creep curve; *i.e.*, a slight decrease of viscosity would be expected even without stimulation.

Discussion

General considerations

The creep curve allows some assumptions about the general mechanical properties of the tooth ligament. Generally, the similarity of creep curves in push and pull tests in living and frozen samples indicates that the basic properties of the tooth ligament do not depend on the direction of load application or on the existence of living cells. The high creep rate at the beginning indicates that the ligament contains elements with low viscosity that relax quickly after a load is applied. The constant creep rate in the second phase is caused by elements with a comparatively high viscosity that relax slowly. The gradually increasing creep rate in the third phase might be explained by the decreasing area of contact between tooth and jaw due to shift of the tooth. However, the tooth shifted on average only 4.7% at failure; the high increase in creep rate can not be explained by the shift of the tooth alone. It is probable that elements of the ligament lose contact after shifting for a comparatively short distance.

Loss of contact results in loss of friction and accelerating creep rate.

The tooth is connected to the jaw by collagen fibrils. The average shift of the tooth (460 μm) is more than the calculated maximal length of the collagen fibril bundles (about 150 μm). Since collagen fibrils cannot elongate more than 10% without rupture (Mason, 1964), the shift of the tooth cannot be explained by strain of the collagen fibrils. Rather, we think that the collagen fibrils or the fibril bundles slowly slide past each other while the tooth is shifting. This conclusion is supported by the observation that collagen is left on both tooth and jaw after the tooth is pulled out. The collagen fibrils probably have no stable bonding, as indicated by our finding that the tooth was extracted in all experiments. The sliding collagen fibrils would lose contact with each other when the tooth has shifted for some distance. This loss of contact results in an accelerating creep rate, as described above.

The constant creep rate in the second phase allows calculation of average shear viscosity of the ligament. With an average creep rate of 6.8 μm/h and average ligament dimensions of 11.7 mm × 600 μm × 24.8 μm (length × width × thickness) the shear viscosity is calculated to be about 550 MPa · s. The dermis of the sea cucumber *Actinopyga echinites* has a tensile viscosity of 100 MPa · s (Motokawa, 1984b). For viscoelastic polymers, tensile viscosity is three times higher than shear viscosity. A shear viscosity of 550 MPa · s in the tooth ligament thus corresponds to a tensile viscosity of 1650 MPa · s, which is one order of magnitude higher than the viscosity of sea cucumber dermis. This finding is reasonable because the tooth ligament of sea urchins must provide a firm connection between tooth and jaw, and thus it may well be much stiffer than sea cucumber dermis.

Variability of mechanical properties

The growing end of the sea urchin tooth is covered by an epithelium that contains some muscular elements. The muscle cells are embedded in the epithelium and arranged parallel to the length axis of the tooth (Birenheide and Motokawa, 1996). Contraction of these cells might push the tooth outward and thus increase the creep rate in pull tests and decrease the rate in push tests. Our results, however, showed that KASW or ACh decreased the creep rate in both tests. Thus the observed effects of ACh and KASW cannot be explained by contraction of the epithelial muscles. The ligament contains only a few cell processes that probably cannot produce any force (Birenheide and Motokawa, 1996). We thus conclude that the observed change in mechanical properties depends solely on changes in the mechanical properties of the extracellular matrix of the tooth ligament.

Acetylcholine (ACh) is a ubiquitous neurotransmitter in echinoderms (Cobb, 1987). Its stiffening effect on the tooth ligament strongly suggests that the mechanical properties are under nervous control. Freezing and rethawing of samples presumably destroys all living cells in the ligament. Lack of any reaction to ACh in frozen ligaments indicates that the stiffening reaction depends on the existence of living cells and is not just a chemical reaction of the extracellular matrix to ACh. Seawater with high potassium content (KASW) also had a stiffening effect only on living ligaments. KASW is a potential stimulator of living cells by depolarizing the cell membrane; here it probably exerted its effect on cellular elements of the tooth ligament. As we have demonstrated (Birenheide and Motokawa, 1996), cells that send processes into the tooth ligament are in contact with a jaw nerve. We think that ACh and KASW influence these cells or the axons of the nerve.

The stiffening effect of KASW on intact ligaments was apparent in 15 min. In frozen ligaments, KASW had a much slower and opposite effect: it accelerated the creep rate within 1 h. Because living cells are presumably destroyed in frozen ligaments, the reaction is probably due to a direct effect of potassium ions on the extracellular matrix. It has been suggested that divalent calcium ions provide crosslinks between proteoglycans of the extracellular matrix, thus stiffening the tissue (Motokawa, 1984a). If calcium ions are replaced by monovalent potassium ions, crosslinking between proteoglycans would be weakened and the tissue would soften.

The effects of ACh and KASW on living and frozen ligaments are strikingly similar to those on the dermis of sea cucumbers (Motokawa, 1994). Living dermis stiffens and Triton-extracted dermis softens after application of KASW. ACh causes stiffening of living dermis, but has no effect on Triton-extracted dermis. Sea cucumber dermis is a composite material consisting of an extracellular matrix with interspersed collagen fibrils. Cellular elements spread throughout the extracellular material (Motokawa, 1982; Matsuno and Motokawa, 1992). In mechanical tests, sea cucumber dermis was shown to be a typical catch connective tissue (CCT) (Motokawa, 1981). CCT is unique to echinoderms and can change its mechanical properties under nervous control (Motokawa, 1984a; Wilkie, 1984). During the stiff state, weak bonds are thought to connect the collagen fibrils and thus prevent them from slipping; proteoglycans are probably involved in the bonding. By some unknown mechanism, nervous signals would induce loosening of the bonds and thus allow slippage of the collagen fibrils. Our study indicates that the tooth ligament is another example of CCT. Variability of mechanical properties is further supported by the high variation in experimental time until failure (25 min to 67 h). Probably our samples were in different states of catch at the beginning of the experiments.

In a preliminary study, Birenheide (1990) showed that papain loosens the tooth ligament dramatically. Papain degrades proteoglycans only, without affecting collagen (Junqueira *et al.,* 1980; Hardingham, 1981). This finding demonstrates convincingly that proteoglycans are responsible for the stiffness of the tooth ligament.

Driving force of tooth shift during natural growth

The presence of muscles in the coelomic epithelium of the plumula seems to have no effect on the mechanical properties of the tooth ligament. If the muscles were connected to the tooth by means of basement membrane and connective tissue, their contraction might provide the force that drives the tooth during growth. This idea is unlikely, however, because the coelomic epithelium is too delicate to provide a solid structure for anchoring the muscles. The tooth itself is pliant in the plumula region and would not transmit forces effectively. We thus do not think that the epithelial muscles of the plumula are the source of the driving force for the tooth in growth. Among other possibilities is a force generated by the newly synthesized tooth elements in the plumula or by the tooth ligament. As in the case of the epithelial muscles, a force generated in the plumula would lack a solid structure as counterpart, but a force generated by the tooth ligament is a probable candidate. Such a force has been postulated for the periodontal ligament of rat incisors (Kirkham *et al.,* 1993). Although there are no data about driving forces for tooth growth in sea urchins, the tooth ligament should be considered as a possible source.

Catch mechanism in tooth ligament

Our data indicate that the dilemma of the echinoid tooth ligament—to be stiff for firm connection of the tooth or to be pliant to allow growth of the tooth—is solved by using catch connective tissue. To summarize our findings, we propose the following model. During feeding the tooth ligament is in a stiffened state. Proteoglycans connect stationary collagen on the jaw side and movable collagen on the tooth side. The connection prevents collagen fibrils from slipping. In this state the tooth is firmly fixed to the jaw and the ligament withstands strong forces when the tooth scrapes over rocks. When the sea urchin is not feeding, a nervous signal causes the connections between collagen fibrils and proteoglycans to loosen. The collagen fibrils can slip past each other and allow slow shift of the tooth during growth. When the sea urchin starts feeding, another nervous signal stiffens the ligament again.

The sea urchin tooth ligament is a convincing example of the use of catch connective tissue to connect skeletal ossicles that need firm connection as well as movability.

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Mutable Collagenous Structure or Not? A Comment on the Re-interpretation by del Castillo *et al.* of the Catch Mechanism in the Sea Urchin Spine Ligament

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It is now widely recognized that one of the characteristic features of the phylum Echinodermata is the presence of collagenous tissues that can undergo rapid, nervously mediated changes in mechanical properties (1,2). Whilst examples of such mutable collagenous tissues have been demonstrated in the five major echinoderm classes (3,4), research over the past 30 years has focused on a certain few of them that are easily accessible and amenable to experimental investigation. The capsular ligament ("catch apparatus"—CA) of the echinoid spine–test joint is one of these well-studied collagenous structures. Because the CA can undergo reversible changes in passive stiffness, it was believed to be a catch muscle by von Uexküll (5). Subsequently it was shown to consist predominantly of collagenous connective tissue with only a scattering of small muscle fibers. Analysis of its mechanical properties and physiological responses led most workers to the conclusion that the CA is a posture-locking device whose functioning depends almost entirely on the variable tensility of its extracellular connective tissue (6–11). However, del Castillo et al. *(12) have presented a model in which its catch properties are attributed only to the contractile cells and in which connective tissue mutability plays no role. The aims of this contribution are to evaluate the new model, to show that it cannot explain the behavior of the CA, and to demonstrate that the extracellular matrix must be the primary source of the catch properties of the CA.*

Organization of the catch apparatus (Fig. 1)

The CA of *Eucidaris tribuloides,* the species studied by del Castillo *et al.* (12), is composed mainly of parallel aggregations of striated collagen fibrils very similar in banding pattern and periodicity, and in biochemical characteristics, to mammalian type-I fibrils (13–15). The fibrils are discontinuous, that is, they are shorter than the overall length of the ligament, and they are arranged in small cylindrical bundles (*i.e.,* fibers, termed by del Castillo *et al.* "columns"), each of which is 2–4 μm in diameter and surrounded by a loose meshwork of filaments of diameter 10–20 nm. Within each small fiber (*i.e.,* bundle of fibrils), there are structural linkages between the fibrils, some of which are proteoglycans attached to the fibrils at regular intervals, again as in mammalian collagenous tissues (12,13,16).

The small fibers are themselves grouped into large fibers of diameter 20–30 μm that, at least in the outer region of the CA, are continuous from insertion to insertion and, for most of their length, strictly parallel to the longitudinal axis of the ligament. Near the insertion of the CA into the test and spine base, small fibers cross between adjacent large fibers, so that the large fibers effectively anastomose at the insertions. Large fibers resulting from the exchange of small fibers enter pores in the skeletal ossicles and at regular intervals within the ossicles branch to form "straps" that loop over the surface of a skeletal bar and join adjacent large fibers (12,16).

In addition to the aforementioned extracellular components, the CA contains muscle fibers 0.1–1 μm in diameter and of undetermined length that are parallel to the collagen fibrils and occupy about 1.5% of the cross-sectional area of the CA. At certain points, possibly at either end of the muscle cells, they are closely appressed to small collagen fibers, the muscle cell being enclosed within the filament meshwork surrounding the collagen fiber at this level (12).

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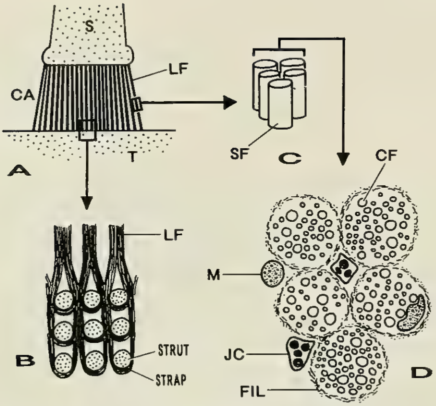

Figure 1. Organization of the CA of *E. tribuloides* (based on micrographs in refs. 12, 13, and 16). (A) Diagrammatic lateral view of a spine–test joint after removal of the spine muscle. CA, catch apparatus; LF, large fiber; S, spine; T, test. (B) Near the insertions the large fibers anastomose and within the ossicle branch to form straps that wrap round skeletal struts. (C) Each large fiber is a bundle of small fibers (SF). (D) Each small fiber is delimited by a meshwork of filaments (FIL) and consists of a parallel aggregation of collagen fibrils (CF). Intraligamental muscle cells (M) and juxtaligamental cells (JC) are also present.

Conventional view of catch apparatus functioning

The spine–test articulation of echinoids is a ball-and-socket joint. The joint is enclosed by the inner cylinder of the CA, and by an outer ring of contractile tissue—the spine muscle. The mobility of the spine depends on two factors: the contractile activity of the spine muscle and the stretch resistance of the CA to external forces. The spine is moved by contractions of the muscle while the CA is in a low-resistance condition. The posture of the spine is frozen when the CA switches to a high-resistance state, presumably accompanied by relaxation of the spine muscle, though this point has still to be confirmed.

Changes in the resistance of the CA to externally applied forces have usually been ascribed to changes in the mechanical properties of the collagen fibers. This was first postulated by Takahashi (7,8), and then appeared to be confirmed by the quantitative data of other workers. For example, Hidaka and Takahashi (9) found that in stress-strain tests the maximum tension that could be sustained by the acetylcholine-treated CA of *Anthocidaris crassispina* was 20 MPa greater than the equivalent for epinephrine-relaxed ligaments. The muscle cells in the CA of *A. crassispina* occupy about 3% of the cross-sectional area. Had isometric contraction of the muscle cells alone been responsible for such a change in the stretch resistance of the whole CA, they would have had to generate an active tension of over 600 MPa. This is clearly absurd, since the maximum tension reported for any muscle is 1.4 MPa (molluscan ABRM), and it indicated that a non-muscular mechanism must bring about the change in CA stiffness.

It was also observed by Hidaka and Takahashi (9) that in transverse sections of the elongated CA of *A. crassispina,* there were fibril-free gaps within the small fibers, which suggested that stretching of the CA involved interfibrillar slippage. The variable stretch resistance of the CA was thus ascribed to variations in the strength of the linkages between adjacent fibrils, as had been deduced for other echinoderm collagenous structures, such as the ophiuroid intervertebral ligament (17) and crinoid syzygial ligament (18).

Regarding the function of the intraligamental muscle fibers, it was reported that when a spine is inclined, so that the CA is elongated on one side and compressed on the other (see Fig. 3), the CA on the compressed side is not kinked or folded. This implied that CA shortening also involves interfibrillar slippage, and it was suggested that the necessary "telescoping" effect resulted from the contraction of the intraligamental muscle fibers (8).

The model of del Castillo et al.

The model depends partly on certain premises concerning the organization of the CA, which the authors derive from their ultrastructural observations. Firstly, they regard the small fibers as being discontinuous, stable units within which the fibrils are strongly bound by a variety of interfibrillar connections, so that changes in CA length take place by means of adjacent small fibers shearing past each other. Secondly, they assume that the extremities of the muscle cells are bonded to the small fibers. Thirdly, they believe that each muscle cell is bonded to different adjacent small fibers rather than to the same small fiber.

According to the new model (Fig. 2), the resistance of the CA to external forces depends ultimately on friction between the ligament straps and the skeletal struts at the CA–ossicle junctions. Having noted that in scanning electron microscope images the straps can be either closely applied to, or detached from, the surface of the skeletal struts, the authors believe that in a "slack" (low-resistance) ligament, the muscle fibers are relaxed and the insertion straps are loosely applied to the struts. To achieve the catch (high-resistance) state, the muscle fibers contract. This exerts a tensile force on adjacent small collagen fibers, and at the CA–ossicle junction pulls the straps tightly against the struts, thus preventing slight movement between the two which is believed to be the basis of the slackened state. The authors estimate that there are about 30,000 strap–strut contacts below each 1 mm^2 of insertion surface and state that "the resistance generated at single strap–strut contacts will be greatly amplified by the multiplicative effect of friction sites in series."

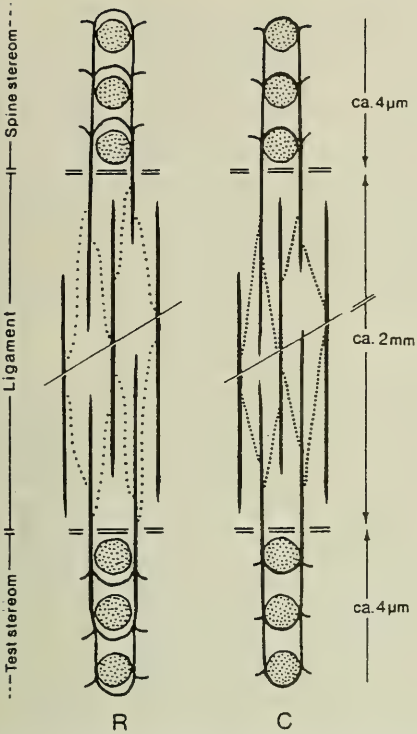

Figure 2. Schematic diagram produced by del Castillo *et al.* (12) to illustrate their new model, showing the "relaxed" ligament (R) and "contracted" ligament (C). The authors assume that the small fibers (solid parallel rods) are discontinuous and that adjacent small fibers are connected only by muscle cells (stippled lines). It should be noted that the latter is impossible, since it would result in the muscle cells having to sustain the whole of any load that was being applied to the ligament. It is known, however, that the tensile strength and stiffness of the ligament are close to those of mammalian tendon (see text).

This interpretation thus admits no role whatsoever for connective tissue mutability in the functioning of the CA.

The new model does not explain the catch phenomenon

At this stage there is a need to recall the essential elements of the catch phenomenon as manifested by the CA. When a spine–test joint is in the mobile condition, the spine can be inclined by the differential contraction of the spine muscle on one side. To allow this to happen, the CA must stretch on the opposite side (see Fig. 3). As is acknowledged by del Castillo *et al.*, this can occur only by the elongation of the large fibers. When a joint is immobilized in the catch condition, the CA has a high stretch

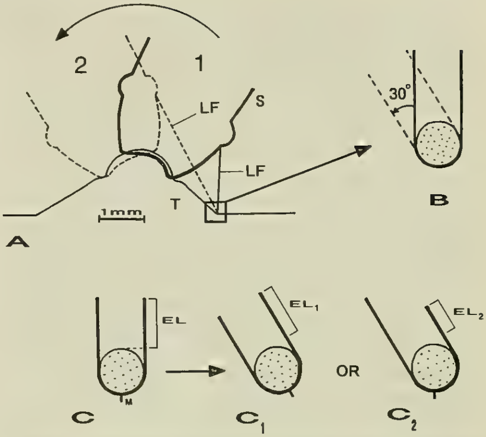

Figure 3. The possible contribution to CA elongation of circumferential slippage between straps and struts. (A) Outline of the ball-and-socket joint of *E. tribuloides* with the spine in two positions of maximum inclination. Position 2 was traced accurately from a scanning electron micrograph (ref. 12), and position 1 was inferred. LF represents a large fiber at the outermost surface of the CA. When the spine moves from position 1 to position 2, this fiber elongates by *ca.* 1.5 mm. (B) Schematic diagram of one ligament loop and its relationship to a skeletal strut in the outermost layer of the test. During the spine inclination illustrated in (A), the large fiber swings through an angle of *ca.* 30°. (C) According to the new model, in a slack ligament the straps can slide over the struts (C_1) (M = marker adhering to the outer surface of the strap). Assuming that the straps form inextensible loops (which is necessary to permit any calculation, but unlikely given that the rest of the fibers are extensible in the slack ligament!), fiber inclination would not change the effective length (EL) of their free arms ($EL_1 = EL$). If, as is proposed by del Castillo *et al.*, in the catch state circumferential slippage is blocked (C_2), inclination of the fibers would shorten one arm of the loop and lengthen the other; since displacement along the axis of the fiber is limited by the shorter arm, the effective length would be reduced ($EL_2 < EL_1$). The contribution of circumferential slippage to fiber elongation is thus ($EL_1 - EL_2$). This can be calculated for the example in (A) above. If circumferential slippage is blocked, an inclination of 90° would change the lengths of the free arms of the loops by the equivalent of one quarter of the circumference of the curved surface of the strut:

$$0.25 \times \text{circumference} = 0.25 \times 3.14 \times \text{diameter} = 0.785d.$$

For an inclination of 30°, the change in length is therefore

$$0.785d \div 3 = 0.262d.$$

From the published micrographs of del Castillo and co-workers (12, 16), I estimate that $d = ca.\ 10\ \mu m$, and so for an inclination of 30° the change in length, *i.e.*, the contribution of circumferential slippage to fiber elongation, is

$$10 \times 0.262 = 2.62\ \mu m.$$

Since, during this maneuver, the large fiber elongates by a total of 1.5 mm, the contribution of circumferential slippage is

$$(2.62 \div 1500) \times 100 = 0.17\%.$$

This must be regarded as a rough, "order of magnitude" calculation. It applies only to those loops lying in a plane that coincides with the plane of movement of the spine, and it ignores events at the other end of the ligament fibers, *i.e.*, at the opposite insertion.

resistance to external forces because the large fibers cannot elongate. Any hypothesis for the catch mechanism must explain how elongation is prevented.

Before expounding their model, del Castillo *et al.* discuss ligament length change and their view that it depends on sliding movements between adjacent small fibers ("columns"). If this view is correct, the only way elongation of the large fibers can be prevented is by the strengthening of the cohesive forces linking together adjacent small fibers. The new model fails completely to address this point and hinges on the concept that the stretch resistance of the CA is influenced by small movements between insertion straps and struts to such an extent that the elimination of these movements by itself brings about the catch condition. However, there is no possible mechanism by which the prevention or facilitation of such *movements* within the insertions could modulate *cohesive forces* within the large fibers; the cohesive forces are not a function of insertion movements. Could these movements affect elongation of the large fibers by some other means?

Unfortunately, del Castillo *et al.* do not specify exactly what kind of movements they are thinking about: their diagram (Fig. 2) shows only a closing of gaps between straps and struts by a pulling force acting normal to the insertion surface. As no data are provided on the width of these gaps, the significance of this type of movement cannot be evaluated. However, this may be irrelevant. Since they refer to "movement of the straps over the struts" and "friction between two sliding surfaces," it appears that they mean circumferential slippage of the straps round the struts. It is possible that such circumferential slippage occurs at least at the level of the most superficial layer of struts when a spine is inclined. However, Figure 3 indicates that when a spine inclines from one extreme position to another, circumferential slippage around the most superficial layer of struts is likely to contribute less than 1% to the extension of the large fibers. Moreover, the presence of several (up to six) layers of struts would not amplify this contribution for the following reason. Any slippage at the insertions resulting from deflection of the spine (by the spine muscle or an external agent) would generate a tensile stress in the large fibers and stress would be transferred from them to the insertion straps. Since it is the case that in a relaxed ligament the fibers are extensible, stress applied by external forces will be dissipated partly in fiber elongation, and stress transfer to the insertions will be reduced correspondingly. There will be a progressive diminution of stress transfer from superficial to deeper levels of the CA–ossicle junctions and a decreasing likelihood that slippage will occur. It has to be concluded that movements between the straps and struts can make no significant contribution to the extension of a relaxed CA, and therefore the prevention of circumferential slippage can make no significant contribution to the catch condition.

This emphasizes that, if the large fibers are assemblies of discontinuous small fibers, the stretch resistance of the CA must depend overwhelmingly on the extent to which shear can occur between the small fibers. The modulation of inter-fiber shear could not be a function of the muscle cells alone, since, as Hidaka and Takahashi realised (9), to bring about catch these would have to generate an impossibly high tensile force. It would have to depend largely or wholly on the physiological control of purely extracellular cohesive forces between the small fibers. In other words, the CA must be a mutable collagenous structure.

Other evidence for the new model

In support of their model, and in addition to their SEM observations of strap–strut relations, del Castillo *et al.* adduce two other areas of evidence. The first concerns the regular geometry of the CA–ossicle insertion regions with the ladderlike arrangement of ligament loops penetrating deeply into the ossicles (Fig. 1). This is supposedly responsible for the "multiplicative effect." The authors noted that such an arrangement is not found at the insertions of the central spine ligament that runs between central points in the articular surfaces of the spine base and condyle of *E. tribuloides.* This ligament lacks intraligamental muscle cells and shows no responsiveness to acetylcholine. However, deeply penetrating fibers looping round successive layers of skeletal struts in a regular pattern are commonly seen at the ligament–ossicle junctions of confirmed mutable collagenous structures that lack intraligamental muscle fibers, *e.g.,* the cirral ligament of crinoids (18), intervertebral ligament of ophiuroids (19), and aboral brachial ligament of crinoids (20). This anatomical arrangement is thus not correlated with the presence of intraligamental muscle cells. It appears to be a design for resisting high tensile loads through the maximization of stress distribution, although another possibility is that, by increasing the length of ligament fibers without increasing the separation distance between insertion surfaces, it provides a spatially economic means of boosting ligament extensibility and resilience.

The second area of evidence pertains to the pharmacology of the CA. This has been investigated by means of a forced vibration technique that reveals changes in passive stiffness ("catch") (21,22), and by static tests in which active force development ("contracture") is recorded (23). It has been shown convincingly that both the pharmacological sensitivity and the time course of "catch" and "contracture" are virtually identical, and it has been argued that both types of response can therefore be attributed to the activity of the muscle fibers (23). However, caution is required here. First of all, if, as I contend, the CA does consist of mutable collagenous tissue, this will be innervated (*via* the juxtaligamental cells: see below and Fig. 1), and so pharmacological effects on passive stiffness could be due partly or wholly to influences on the connective tissue rather than the muscle cells. Secondly, it remains to be demonstrated that the "catch" response reported by del Castillo and co-workers is quantitatively equivalent to the variable tensility of the CA described elsewhere in the literature. Del Castillo *et al.* have not attempted to quantify what they refer to as "catch" in *E. tribuloides,* but their published recordings from experiments on whole CA preparations show active force development and changes in passive resistive force that are in the order of a few (< 10) gf. From data in Vidal *et al.* (23) and other papers, I estimate that the whole CA can generate a maximum active tension of 5 KPa (*e.g.,* in response to 100 μm acetylcholine), which, if the muscle cells occupy 1.5% of the cross-sectional area, would require them to generate a tension of 0.3 MPa. It has already been mentioned that the maximum tension that can be resisted by an acetylcholine-stiffened CA is 20 MPa greater than the equivalent for an epinephrine-relaxed CA (9). Employing the CA of *E. tribuloides* itself, Szulgit and Shadwick (11) showed that simple ionic manipulation can vary the tensile strength from 2–15 MPa. It is thus clear that the changes in passive mechanical properties that have signified the occurrence of variable tensility in the CA are probably more than 1000-fold greater than the active tensions recorded by del Castillo and co-workers. Indeed they recognized that "a powerful mechanical amplifying system" (23) would have to be present to explain how weak "contractures" could be converted into strong "catch."

Conclusions

In the catch state the CA has a tensile strength of *ca.* 30 MPa and a tensile stiffness of *ca.* 300 MPa (9), values that are close to the equivalents for mammalian tendon (20–140 MPa and 1 GPa respectively) (24). Out of catch, the CA is highly compliant: it can be stretched easily by the action of the spine muscle and *in vitro* creeps to rupture under low loads like a viscous fluid (7). Such huge changes in mechanical properties are commonplace in echinoderm mutable collagenous structures, many of which have an internal organization very similar to that of the CA, and most of which lack intraligamental muscle cells (3,4). The latter fact alone would make it highly unlikely that catch in the CA depended on the muscle fibers, because it is not credible that different echinoderm collagenous structures have evolved catch properties underpinned by entirely separate mechanisms. In addition, the CA is densely supplied with granule-containing juxtaligamental cell processes (Fig. 1) belonging to perikarya that come into close contact with the nervous system (25). In echinoderms, the occurrence of juxtaligamental elements is correlated very strongly with the capacity for variable tensility: they have been found in all confirmed mutable collagenous tissues (4), but are absent from echinoderm collagenous structures that lack the capacity for variable tensility (the nonautotomy tendons of an ophiuroid (26), compass-rotular ligaments of a sea urchin (27), and central spine ligament of *E. tribuloides* (12)). The juxtaligamental cells in the CA are highly suggestive of the presence of mutable collagenous tissue (MCT). Thus there are *a priori* arguments as well as experimental evidence supporting the view that the CA is a mutable collagenous structure, all of which is ignored by del Castillo *et al.*

Despite its inadequacies, the new model serves a useful purpose in highlighting some problematical aspects of the biology of the CA and MCTs in general. There is need for more information on their extracellular organization (*e.g.,* to pinpoint which are the sliding elements), on the identity of their interfibrillar components, and on the nature and properties of the links holding these together. Intraligamental muscle fibers are absent from the vast majority of MCTs, and so their presence in the CA is intriguing. Other MCTs can reshorten without them and so must have an internal mechanism for strain recovery (which may be the function of the filament meshworks); possibly the muscle cells of the CA accelerate its reshortening. Finally, MCT innervation and its pharmacology are still only patchily understood. There is a need to develop pharmacological approaches that, in tandem with morphological analyses, could distinguish mechanical responses due to the MCT itself from those due to the activity of contractile cells in or near it.

The CA will continue to play a crucial role as one of the key experimental subjects for MCT research.

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We Still Invoke Friction and Occam's Razor to Explain Catch in the Spines of *Eucidaris tribuloides*

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The principle of economy that emerged from the views of William of Occam, namely that where alternative hypotheses exist, the one involving the fewest assumptions is to be preferred, is still valid in science. Indeed, it was the application of "Occam's razor" that led us to propose a new model (1) to explain that mechanism of catch contraction in the primary spines of Eucidaris tribuloides—*the model that I. C. Wilkie (2) considers inadequate through its failure to invoke collagen mutability.*

In the 1960s, Takahashi (3, 4) took up the study of the articular capsule, or ligament, called by him 'catch apparatus' (CA), that surrounds the primary spine–test articulation of the sea urchin *Anthocidaris crassispina.* At that time, this ligament was believed to be a muscle; thus he was surprised to see that neither acetylcholine (ACh) nor epinephrine exerted detectable effects on its tension during isometric recording. But when the recording was isotonic (creep test), both compounds induced distinctly different effects on the rate of elongation of the preparations. ACh decreased it, whereas epinephrine exerted an accelerating effect. Takahashi recognized the collagenous nature of the CA and concluded that its consistency must be mutable—switching, under the influence of these neurotransmitters, between two extreme conditions or states: one soft, or extensible, and the other stiff, or inextensible.

The idea that the nervous system may exert a direct action on collagen was attractive, and we began working on *Eucidaris* with the aim of elucidating its mechanism. But none of our observations required, as an explanation, that the nervous system have a direct influence on the extracellular matrix. Indeed, the outcome of several approaches led us to conclude that all our results could be explained in terms of a simple mechanical interaction between the collagen and muscle fibers that make up the fabric of the echinoid ligament.

The arrangement of the muscle and collagen fibers in the echinoid articular capsule is very different from that of a simple muscle–tendon–bone sequence. In the ligament, the muscle fibers (which represent only 1.5% of the cross-sectional area) cross-link large collagen fibers (>95%) that end at the insertion surfaces of the spine and the test, forming loops or straps that, when the CA is not in catch, fit loosely around osseous struts, or microbeams, in the stereom. But when the muscle fibers are activated by ACh, the straps are appressed against the struts, and the friction generated by the contact of the two surfaces prevents the movement of the spine. The function of the ligament is to "freeze" the spine, preventing it from moving under the influence of external forces applied to its shaft. Rather than producing work, the ligament is an energy-absorbing or energy-dissipating device, resembling in some respects the hydraulic braking system of a vehicle.

Wilkie's main argument against our model is that, for it to work, the muscle fibers would have to generate "an impossibly high tensile force," and, quoting from our article (1), he adds that we recognize the need for a powerful amplifying system to convert weak contractures into strong catch, implying that such an amplifying system is yet to be identified. But the precise purpose of our paper was to show that friction can easily provide the high amplification needed without requiring an additional power supply!

Perhaps we have not adequately explained our views on the role of friction in catch, and a more graphic depiction of them is in order. If a weight attached to a rope is to be lifted, passing the rope around a horizontal post will confer a mechanical advantage. Thus, if we arrange a number of identical posts in series, and we assume that a single post gives us a mechanical advantage of 10×, a

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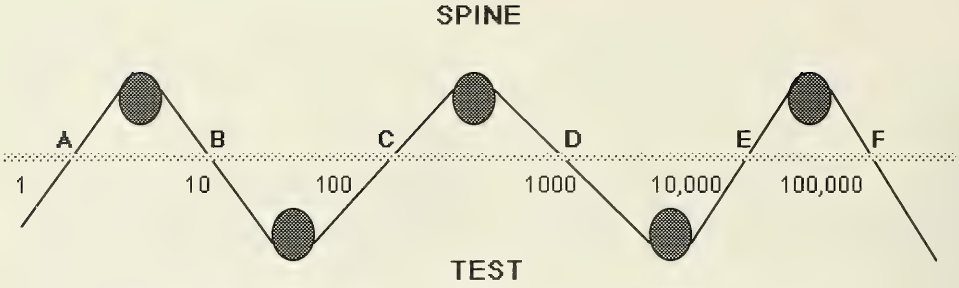

Figure 1. Simplified diagram of the organization of the large collagen fibers in the catch apparatus of *Eudicaris tribuloides*.

pull of 1 g at A will hold 10 kg at E (see Fig. 1). This highly simplified diagram further shows our view of the organization of the CA. Because the multiplicative effect of friction occurs only when the friction sites are in series, it requires the concerted action of sites on both sides of the spine–test articulation. We must emphasize that while we are fully aware of the complex and redundant disposition of the collagen fibers in the CA, there is no reason why our Figure 1 should not be regarded as an equivalent "wiring" diagram of the ligament.

An important feature of the CA design seems to be that the muscle fibers, which cross-link the collagen columns and are the only source of force in the CA, are arranged in parallel with the large collagen fibers; thus the slender muscle fibers (0.1 to 1.0 μm in diameter) are protected from ever being exposed directly (*i.e.*, in series) to the high tensile forces that they themselves generate and that would tear them apart.

The existence of mutable connective tissues in other echinoderm preparations is used by Wilkie as another argument in support of his views and is something we do not disclaim. Indeed, as we pointed out (1), our conclusions from the spines in *Eucidaris* are not to be extrapolated to other echinoderm systems. We suggest that while MCTs may well have evolved to perform some mechanical tasks in some echinoderms, evolution has solved the need for a 'catch' in the primary spines of *Eucidaris* in a simpler and highly efficient manner. Wilkie fails to offer a solid alternative mechanism, or counter-evidence to explain our results. He only suggests that catch could be explained as an increase of the cohesive forces that keep the large collagen fibers together. This is a reasonable supposition, which we also entertained briefly, but that so far lacks experimental support.

Although Wilkie regards the CA as one of the "well-studied collagenous structures," it is obvious from our papers that further work on this system is still needed to clarify problematical aspects of the biology of echinoderm tissues in general.

Acknowledgments

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Toward a National Agenda for Research in the Coastal Zone: Where Are We?

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> "It is no longer obvious that the organizational structures of either federal or nonfederal institutions are as effective as they might be. . . . only one prediction is secure: the ways in which science in the United States will be conducted and supported in the next several decades will differ from the ways to which we have become accustomed." (Wunsch, 1993)

Abstract. In response to the complex challenges of understanding and managing the effects of human activities on coastal ecosystems, the research establishment has produced a plethora of reports and implemented a bewildering array of government-sponsored research and monitoring programs that are not cost-effective on regional or national scales. Consequently, the gap between national needs and the capacity of the nation's research institutions to respond is growing rapidly, and environmental policies are ambiguous at a time when coastal habitats and living resources are being lost or degraded at accelerating rates. A case is made that a grassroots effort is needed to develop cost-effect programs that are responsive to both local needs and national priorities.

Introduction

The effects of human activities on the environment and natural resources are expected to be especially pronounced in the coastal zone as a consequence of demographic trends and global climate change. Nearly 50% of the U.S. population lives in coastal counties that make up 10% of the nation's land area and account for at least 30% of the nation's GNP (Culliton *et al.*, 1990). Although continental shelves, the Great Lakes, estuaries, lagoons, and bays constitute less than 10% of the earth's surface, they account for 25% of global biological production and support over 95% of its fisheries. The U.S. Department of Commerce (1992) reports that the cumulative impact of commercial fisheries on the nation's GNP is over $50 billion. Annual revenues from recreational fishermen and boaters and from oil and gas deposits in coastal waters account for $23 billion and $16 billion, respectively (Council on Environmental Quality, 1993). At the same time, rapid population increases and related human activities in coastal watersheds have altered fresh water flows (surface and ground water), increased shoreline erosion, added excessive nutrients and toxic chemicals, introduced nonindigenous species, and depleted natural resources. These trends have been associated with increases in the susceptibility of coastal communities to natural disasters, losses of habitat, eutrophication, toxic algal blooms, fish kills, and public health problems, all of which threaten the quality of life and the economies of coastal states and the nation (NRC, 1994a).

Given the magnitude and critical nature of these problems, nationally coordinated and locally relevant programs of research and monitoring are manifest national needs. Here we discuss the challenges that must be met to successfully effect such programs in the coastal zone. This report is the product of a workshop organized by the National Association of Marine Laboratories (NAML), the purpose of which was to recommend mechanisms by which the community of coastal scientists can more effectively work together and with government agencies to define priorities and to implement programs that are responsive to national needs, cost-effective, and locally relevant. As a first step, long-range plans and recommendations of federal agencies and science advisory groups (Table I) provided the basis for (1) identifying central re-

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This paper was originally presented at a workshop titled *Roles of Coastal Laboratories in the Implementation of the Nation's Emerging Priorities for Research in the Coastal Zone.* The workshop, organized by the National Association of Marine Laboratories, was held in Sarasota, Florida, from 24 to 27 October 1995.

search themes, (2) reviewing the status of the research enterprise in the coastal zone, and (3) improving our understanding of the forces that will shape the efforts of coastal laboratories as they plan and implement programs of research and monitoring.

Research Themes for the 1990s and Beyond

In 1991, the Ecological Society of America challenged the scientific community with the "Sustainable Biosphere Initiative" (SBI) (Lubchenco *et al.,* 1991). Global change, biological diversity, and sustainable ecosystems were identified as priority areas of research based on their importance for the advancement of fundamental knowledge needed to manage for a sustainable biosphere. The SBI calls for research on global change that emphasizes the underlying importance of species and habitat diversity, distribution patterns of ecological assemblages, and differences in the production and storage of organic matter in terrestrial and aquatic systems.

The broad environmental problems defined by the SBI have been embraced in subsequent recommendations and long-range plans for research in the coastal zone (JOI, 1990; CENR, 1994; Naiman *et al.,* 1995; NRC, 1992, 1994a, b, c, d, 1995a; NSF, 1992, 1993; NOAA, 1993, 1995a). In its 1992 report (NRC, 1992), the Ocean Studies Board (OSB) concluded that determining the role of the ocean in climate change and in the dynamics of coastal ecosystems is among the highest priorities for the ocean sciences. The study highlighted the importance of interdisciplinary approaches to developing a predictive understanding of the interplay between ecosystem processes and global-scale processes and change. More recently, the Committee on Environment and Natural Resources (CENR) (NRC, 1994a; CENR, 1995) identified the following problems as high priorities for research in coastal ecosystems:

- eutrophication, habitat modification, and shoreline erosion;
- hydrologic and hydrodynamic disruption of ecosystems;
- exploitation of resources and introductions of nonindigenous species;
- effects of toxins and pathogens on the health of ecosystems, populations and people; and
- global climate change and variability.

These important problems are, for the most part, reflected in plans adopted by the boards of the Regional Marine Research Program (RMRP, 1993).

The CENR emphasized the importance of biodiversity and the significance of these factors in reducing biodiversity. A subcommittee of the CENR on biodiversity and ecosystem dynamics (NRC, 1995a) recommended that regional strategies of monitoring and research be implemented with the following objectives: (1) understand the patterns, processes, and consequences of changes in biological diversity in marine ecosystems; (2) improve linkages between marine ecology and oceanography; (3) strengthen and expand the field of marine taxonomy; (4) promote the use of new technologies (*e.g., in situ* sensors and molecular techniques), predictive models, and retrospective analyses; and (5) use advances in the understanding of causes and consequences of changes in biodiversity on the regional scale to improve predictions of the impacts of human activities on the marine environment. The CENR reports highlight the importance of watershed models (from drainage basin to the coastal ocean), predictive ecology, and comprehensive ecosystem management.

The need for new and more integrated approaches to the problems of environmental change and biodiversity in coastal ecosystems is international in scope. The Land-Ocean Interactions in the Coastal Zone (LOICZ) program was initiated to determine the effects of changing patterns of climate, sea level, land-use and other human activities on the fluxes of materials between land, sea, and atmosphere (Holligan and de Boois, 1993). The importance of documenting global variability and trends in the marine environment was also acknowledged by the Intergovernmental Oceanographic Commission when it conceived the global ocean observing system (GOOS). The objectives of GOOS are to monitor, assess, and predict changes in climate, living marine resources, and ecosystem health. The coastal module of GOOS is currently in the planning stages (Turgeon, 1995).

The International Marine Biodiversity Program (IMBP), a component of the Ecosystem Function of Biological Diversity Programme (Diversitas) (diCastri and Younes, 1990, 1994; Perry, 1993), was established to understand the linkages between biodiversity and ecosystem function and to "help define the relationships among science, conservation, and management through research undertaken among regional networks of coastal and marine laboratories" (Grassle *et al.,* 1991; Lasserre *et al.,* 1994). A related effort has been initiated by the Inter-American Institute for Global Change Research (IAI). The IAI identified environmental change in the coastal zone as a major priority and sponsored a series of workshops on ocean-land-atmosphere interactions in the inter-tropical Americas, impacts of climate change on biodiversity, and comparative studies of oceanic, coastal, and estuarine processes in temperate zones (IAI, 1995a, b, c). These initiatives would also be effected through a network of research facilities throughout the Americas which will augment research capabilities and promote education and training.

Table I

Recommendations and strategic plans reviewed for this report and published during the period 1992–1995

Scientific Advisory Boards

Publication title	Board	Year
Interim Report of the Committee on Research and Peer Review in EPA	NAS	1995
Comparative Studies of Oceanic, Coastal and Estuarine Processes in Temperature Zones	IAI	1995a
Ocean/Land/Atmosphere Interactions in the Inter-Tropical Americas	IAI	1995b
Study of the Impacts of Climate Change on Biodiversity	IAI	1995c
Science Policy and the Coast: Improving Decision Making	NRC	1995c
Understanding Marine Biodiversity	NRC	1995
Improving Interactions Between Coastal Science and Policy	NRC	1995b
Priorities for Coastal Ecosystem Science	NRC	1994a
Molecular Biology in Marine Science	NRC	1994c
Environmental Science in the Coastal Zone	NRC	1994b
A Review of the National Sea Grant College Program	NRC	1994
A Review of the Accomplishments and Plans of the NOAA Coastal Ocean Program	NRC	1994d
Oceanography in the Next Decade: Building New Partnerships	NRC	1992
A New Horizon for Biological Field Laboratories and Marine Laboratories	OBFS & NAML	1995
Challenges for Limnology in the United States and Canada: An Assessment of the Discipline in the 1990's	ASLO	1995
Changing Expectations and Assessment of the Research University	AAAS	DRAFT
Land-Ocean Interactions in the Coastal Zone Implementation Plan	IGBP	1995
A National R&D Strategy for Water Resources and Coastal and Marine Environments	CENR	DRAFT
Our Changing Planet. The FY 1995 US Global Change Research Program	CENR	1994
Environmental Quality	CEQ	1993
Environmental Research and Development: Strengthening the Federal Infrastructure	Carnegie Commission	1992
Enabling the Future: Linking Science and Technology to Societal Goals	Carnegie Commission	1992

Federal Agency Reports

Title	Lead Agency	Year
The Coastal Module of the US Global Ocean Observing System: A Strategic Plan	NOAA	1995
Sea Grant's Strategic Plan 1995–2005	NOAA	DRAFT
Healthy Coastal Ecosystems and the Role of Integrated Coastal Management	NOAA	1995b
NOAA Center for Coastal Ecosystem Health FY94/95.	NOAA	1994
National Undersea Research Program Strategic Plan for FY1996–2005	NOAA	1994
National Oceanic and Atmospheric Administration 1995–2005 Strategic Plan	NOAA	1993
A Strategic Plan for a Coastal Forecast System	NOAA	1993
Our Living Oceans: Report on the Status of U. S. Living Marine Resources	Department of Commerce	1992
NSF in a Changing World. The National Science Foundation's Strategic Plan	NSF	1995
Global Ocean Ecosystems Dynamics and Climate Change, A Long Range Science Plan 1995–2005	NSF	1995
Eastern Boundary Current Program: A Science Plan for the California Current	NSF	1994
Directorate for Geosciences Long Range Plan FY 1994–1998	NSF	1993
Global Change Research Program	NSF	1993
Long Time Series Measurements in the Coastal Ocean: A Workshop	NSF	1993
Coastal Ocean Processes: A Science Prospectus	NSF	1992
The New Generation of Environmental Protection: EPA's Five Year Strategic Plan	EPA	1994
Spaceborne Synthetic Aperture Radar: Current Status and Future Directions	NASA	1995
Mission to Planet Earth Strategic Enterprise Plan 1995–2000	NASA	1995
Directory of Centers	NIH	1993

Regional Marine Research Programs

Title	Region	Year
Greater New York Bight Research Plan, 1993–1996	Greater New York	1994
South Atlantic and Caribbean Regional Marine Research Plan	South Atlantic and Caribbean	1994
Mid-Atlantic Research Plan	Mid-Atlantic	1994
Insular Pacific Regional Marine Research Program Plan 1992–1996	Insular Pacific	1993
Pacific Northwest Regional Marine Research Program Research Plan 1992–1996	Pacific Northwest	1993
Alaska Regional Marine Research Plan 1992–1996	Alaska	1993
Regional Marine Research Gulf of Mexico Research Plan	Gulf of Mexico	1993
Gulf of Maine Research Plan	Gulf of Maine	

The reports discussed above and listed in Table I underscore the importance of understanding biodiversity and ecosystem dynamics as a basis for sustaining healthy ecological systems and the living resources they support. Global change, biodiversity, ecosystem health, and predictive ecology are recurrent themes. Principal concerns in the coastal zone include:

- the influences of global climate change and pollutant transport on habitats and ecosystems of the coastal zone;
- regional characterization of the diversity of species, habitats, trophic linkages, and biological processes from drainage basins to coastal waters; and
- the "health" of these systems in terms of their capacity to support living resources and assimilate nutrients and contaminants.

Cutting across these themes are the problems of documenting and predicting variability and change over a spectrum of scales in time, space, and ecosystem complexity. Perhaps the greatest and most immediate challenge to the scientific community is the development of robust ecological theories and models that will enable useful predictions of future conditions that can, in turn, be used to test hypotheses and to manage the effects of human activities on the environment.

Roles of Coastal Laboratories

As important as these planning activities are, they have taken place with little regard to the capabilities, facilities, and human resources of coastal laboratories that are and will be needed to implement cost-effective programs of research and monitoring. The costs of research are escalating rapidly (Wunsch, 1993), and the relationship between research laboratories, their faculties, and federal agencies has changed (Ausubel and Steele, 1993). In particular, coastal laboratories are expected to develop and implement relevant, comprehensive, and cost-effective programs of research and monitoring in coastal ecosystems. The success of such an effort will depend a great deal on constructive collaborations among federal agencies and coastal laboratories to (1) overcome parochial attitudes and approaches, (2) develop and coordinate research and monitoring programs that make optimal use of resources, and (3) respond to new demands and expectations.

Parochialism

Coastal laboratories have historically been incubators for creative and innovative science (*cf.*, Wunsch, 1993). For more than a century, these laboratories have played major roles in the development of the natural sciences by setting research priorities, by providing access to coastal habitats and the organisms they support, and by providing the facilities required to study them. Their successes are reflected in the diversity and scope of research activities they support—from biomedical research and molecular biology to coastal circulation and systems ecology. Over the years, some laboratories have expanded their operations to include larger scale explorations of the sea, but most have continued to emphasize local organisms and ecosystems. Consequently, coastal laboratories have tended to operate more or less independently. Given the complexity and comprehensive nature of the research problems discussed above, such independence—and the isolation it often involves—is no longer a viable mode of operation, nor is it conducive to good science. If the laboratories are to play a role in setting priorities and developing comprehensive programs to address them, the history of coastal parochialism must be overcome. The notion that every system is unique, or is uniquely suited for the study of whatever process happens to be currently in vogue, is slowly giving way to the demands of interdisciplinary research, predictive ecology, and environmental management. At the federal level, the conflicts embodied by the mismatch between the structure of the federal bureaucracy and current environmental issues (Carnegie Commission on Science and Technology and Government, 1992) are exacerbated by the federal penchant for both "pork barrel" funding and mandated "global" solutions to local problems.

Mechanisms are needed that provide the means for defining and solving local environmental problems in the context of national priorities. Participants in a recent workshop on the future roles of field stations and marine laboratories (Lohr *et al.*, 1995) recognized this problem and concluded that the networking capabilities of the nation's laboratories should be exploited to (1) optimize the use of resources; (2) provide interdisciplinary approaches to the solution of environmental problems; and (3) promote science education for an informed public. The challenge will be to achieve these goals without losing the "sense of place" and individual innovation that often spawn good science.

Cooperation, coordination, and collaboration

In response to the complex challenges of understanding and managing the effects of human activities on the coastal environment, federal agencies and the scientific community have produced a plethora of reports (Table I) and implemented a large number of research and monitoring programs (CENR, 1995). A review of these efforts reveals a bewildering array of government-sponsored research and monitoring activities that are both incomplete and redundant. Major deficiencies in the nation's research effort have been attributed to a combination of factors (Naiman *et al.,* 1995; CENR, 1995) including (1) the need for more holistic, interdisciplinary approaches to research on systems from organisms to regions; (2) poor definition of priorities and problems; (3) ineffective use of resources; and (4) the lack of national and regional coordination of local research programs from planning to implementation. The CENR report (1995) underscores the urgency of developing "*a national coastal ocean strategy directed at integrating ongoing efforts and promoting the synergy required to tackle multidisciplinary problems that arise from complex societal issues.*"

Responding to national needs

As the complexity and magnitude of environmental problems increase, the costs of the research required to solve them are also going up in both absolute terms and relative to federal funding. The gap between national needs and the capacity of the nation's research institutions to respond continues to grow. Moreover, this is happening during a time of transition, a period when the roles of coastal laboratories and federal agencies are changing (Wunsch, 1993; Lohr *et al.,* 1995; Naiman *et al.,* 1995). A separation is occurring between the setting of priorities (power) and implementation (responsibility).

The balance of power between the federal bureaucracy and research laboratories is shifting as the process of setting priorities and defining research problems becomes increasingly the domain of federal funding agencies (Ausubel and Steele, 1993). At a time when the nation's research laboratories are being told to "do more with less," expectations are growing for greater predictive power, especially for the application of scientific information to the problems of environmental management. Interdisciplinary approaches are creating new demands, not only for measurements on finer time and space scales for longer periods, but also for the integration of data from diverse sources (*e.g.,* atmosphere, drainage basin, and estuary); and new technologies are increasing the volume of data and rate of information exchange. Although coastal laboratories have become less involved in programmatic decisions, the resources required to respond to these demands are, for the most part, in their domain. At the same time, human resources, expertise, and technologies have grown rapidly relative to the nation's investment in the environmental sciences (NRC, 1992).

The difficulties created by the partitioning of power and responsibility are compounded by growing pressures to produce "relevant" results. Understandably, the public, environmental managers, and elected officials are demanding results that are useful in the short term—that solve problems now. At the same time, current fiscal realities require that the scientific community do more with less in the face of the growing complexity and magnitude of the environmental problems that plague modern society.

Conclusions

The issues of global change, biodiversity, and ecosystem health are major concerns that do not recognize bureaucratic or institutional boundaries. For the foreseeable future, research in the coastal zone will be related to the goals of predicting environmental variability and change and to the development of a body of ecological theory that explains relationships between the structure and function of systems and the propagation of variability between large and small scales of organization, *e.g.,* between local and global scales of variability; between molecular genetics and environment; and between biodiversity and ecosystem function. A major effort will be required to develop the intellectual foundations for the formulation of ecologically and economically sound environmental policies. These goals will not be achieved without fundamental changes in how research priorities are determined and programs are organized and funded. As discussed below, cooperation, coordination, and collaboration toward common goals requires certain shared realities.

Although goals and rationales may differ, the means and technologies used to make measurements and acquire data share many common characteristics. Operational goals to develop long-term time-series measurements, interdisciplinary approaches, and regional integration of results are not unique to a particular federal agency or research institution. For example, although agencies and scientists often distinguish between research and monitoring, the distinction is artificial and arbitrary. Regulators and scientists often monitor the same environmental variables, but for different purposes. To the regulator, the goal may be to assess compliance. To the scientist, monitoring is a research activity that reveals patterns in nature. Scientists formulate and test hypotheses with the goals of explaining these patterns and forecasting future states. What is often forgotten or neglected is the fact that the success of both activities—research and monitoring—depends on a constructive interplay between them. We have learned that long-term, high resolution time-series measurements (monitoring) of key environmental and biological variables are required to document and quantify trends (*e.g.,* Vincent *et al.,* 1993; Turgeon, 1995). Regional-scale approaches (*e.g.,* drainage basin–estuary–coastal ocean) promote an understanding of interactions among ecosystems and the power to predict the probability of future states of a given ecosystem (*e.g.,* JOI, 1990; RMRP, 1993). New technologies, especially those that employ molecular methods, *in situ* measurements, and remote sensing, must continue to be developed and used to quantify environmental and biological variability. Hypothesis-driven research and comparative analyses of organisms, coastal ecosystems, and regions will provide the information base for the development of ecological theory upon which comprehensive environmental policies will be founded. Recognition of the fact that these activities are not goals but means to an end that can be shared among agencies and institutions is the first step toward the cost-effective use of limited resources.

Research programs that employ these approaches and technologies depend on the nation's infrastructure of coastal laboratories. However, economic realities, political forces, and resistance to change have made the rational establishment of priorities and the commitment of resources based on long-term needs virtually impossible. It is becoming increasing difficult for individual laboratories to set research priorities and goals and to bring the required resources to bear on complex problems in a timely and effective manner. Historically, coastal laboratories functioned as catalysts for independent approaches and new ideas and provided the means by which priorities were set. But, as the utility of understanding environmental and biological variability in the broader context of ecosystems and regions has become more widely accepted, the power to set priorities has begun to shift to federal agencies, and the tendency to expand the scope of programs has increased. Indeed, the goals of many programs have become so grand that they go beyond the missions and means of the sponsoring agency or department. An important consequence is that the criteria for formulating and implementing effective environmental policies are ambiguous at a time when the degradation of coastal habitats and living resources is accelerating. Clearly, more integrated and comprehensive approaches to understanding and predicting changes in atmospheric, terrestrial, and aquatic systems and related effects are needed (CENR, 1995; NRC, 1995b, c).

Coastal laboratories can play vital roles that have not yet been realized. Their geographic distribution defines the U.S. coast line and provides access to virtually every kind of environment that constitutes the land-sea interface. Coastal laboratories not only make ecosystems and organisms of this important region accessible, they provide many of the facilities and human resources needed to study them. They are vast reservoirs of information about coastal ecosystems and the resources they support. As such, they have the potential of functioning as the nation's coastal sense organs and memory systems.

Coastal laboratories are also in a unique position to address public concerns and to link local, regional, and federal governments in a common effort to define problems and set priorities. Through accelerated exchanges of ideas, information, and new technologies, networks of coastal laboratories could provide the means of (1) promoting scientific programs at local, regional, and national levels to provide information on trends and underlying causes; and (2) facilitating consensus among scientists and stake holders (*e.g.*, industrialists, farmers, fishermen, environmental advocacy groups, and government agencies) leading to ecologically, culturally, and economically sound environmental management.

The realization of relevant and cost-effective research in the coastal zone will depend on the ability of laboratories and government agencies to share resources and form coalitions whose resources are tuned to the problems at hand—to define research priorities and goals; to provide the resources needed to achieve them; to coordinate the implementation of programs, to exchange data and information; and to share facilities and expertise.

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Regional Coastal Research—What Is It? Why Do It? What Role Should NAML Play?

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Abstract. Regional coastal (*i.e.*, marine and Great Lakes) research is an increasingly popular, but still ill-defined, activity. The purpose of regional research and monitoring is to help us understand how events and processes at higher levels of organization, over larger spaces and longer times, influence local ecosystems of interest or concern. Research at this scale will require us to work in larger multidisciplinary and multi-institutional teams than we traditionally have. A survey of papers published in *Estuaries*, *Continental Shelf Research*, and *Deep Sea Research* confirms the impression that estuarine studies are more commonly carried out by one or two investigators, and that those investigators are commonly from a single institution. However, the most important challenge facing coastal ecologists as they embark on larger-scale research lies, not in increasing collaboration, but in their traditional approach to science. We marine ecologists too often indulge in elaborate *post hoc* explanations for the behavior of the complex systems we study, rather than focusing on the development and testing of falsifiable empirical theories. Moreover, we tend to describe our research priorities and accomplishments using fuzzy terms and concepts that seldom have clear operational definitions. As a result, we are in danger of losing the confidence and support of the public and the science funding agencies. It will not do to embark on regional research by simply trying to measure more things in more places more often. The coastal marine research community, perhaps through the National Association of Marine Laboratories (NAML), must reexamine the way it has been doing research. We will have to do smarter science if we are going to prosper in a future of diminished resources.

Introduction

> **region,** n. A part of the earth's surface (land or sea) of considerable and usually indefinite extent.
>
> *Random House Dictionary of the English Language* (unabridged)

A learned judge once said of pornography that he could not define it, but he certainly knew it when he saw it. We are in a similar condition with regard to regional marine research. The term appears with increasing frequency, and there seems to be a growing consensus that we should do more of it (National Research Council, 1994). But I have not found a clear and satisfactory definition of what regional research actually is. Moreover, we should think a bit more about why we feel drawn to study areas of "considerable and usually indefinite extent." The reasons will have to be compelling in a future of diminishing resources.

The United States must contain thousands of "regions" that have been defined in various ways by a great diversity of groups for an enormous variety of reasons. As a result, the country can be seen as a colorful patchwork of everything from telephone area codes to climate zones. The U.S. Congress recently defined nine Regional Marine Research Program (RMRP) areas (Fig. 1), but the boundaries of the regions were never explicitly grounded in terms of natural science, or even administrative expediency. If the National Association of Marine Laboratories (NAML) is going to play a role in developing a regional approach to marine research and monitoring, it could make a useful start by collaborating with federal and state agencies in a thoughtful consideration of the boundaries of such regions. Of course, the description of boundaries should be tied in a fundamental way to our definition of regional

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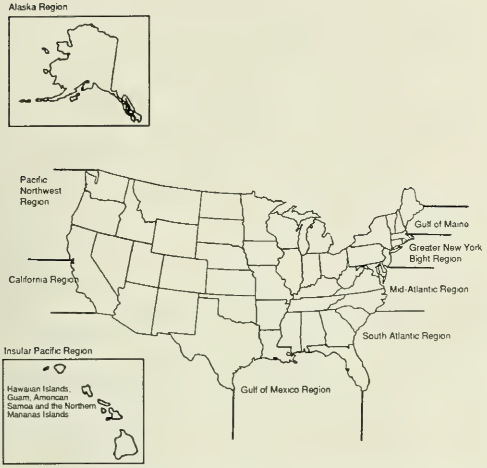

Figure 1. Regional Marine Research Program (RMRP) areas designated by legislation establishing the program. The RMRP is to be administered jointly by the National Oceanic and Atmospheric Administration (NOAA) and the U. S. Environmental Protection Agency (EPA) under authority of the South Carolina Fish Hatchery Act (PL 101-593).

research and to the reasons that lie behind our interest in carrying out research and monitoring on such a scale.

Some Thoughts on Defining Regions and Some Reasons for Doing Regional Research

It may seem odd to begin this section by recounting a lesson on doorbells, but it made a great impression on me over 25 years ago. In his class on systems ecology, H. T. Odum once asked a student to imagine that he was going to visit a friend's house with a stranger from outer space. (It was the late 1960s, and we must allow for some extravagance in teaching methods.) On reaching the house, the student pushed the doorbell. A few moments later, the friend opened the door. All this impressed the space person, and he asked the student how the doorbell worked. Professor Odum asked the student for his response.

Since he had taken at least two semesters of physics, the student delivered a fine description of a basic electrical circuit that would ring a bell when a button was pushed. Dr. Odum asked if anyone wanted to add to the explanation. Another student launched into a discussion of the passage of sound waves through the air. A third provided a sketch of the human inner ear. "All well and good," said Dr. Odum, but we had left out something important. You cannot understand how a doorbell "works" unless you also have some understanding of our concepts of privacy and private property.

Like virtually all traditional science students, we had been taught that understanding comes from a knowledge of mechanisms and processes that operate at a lower level of organization than the phenomenon of interest. Odum's point was that understanding also requires a knowledge of the next higher level of organization. The function of a doorbell is as much a product of social systems and legal conventions as of wires, electricity, and sound waves. In Odum's words: "We must study the world with a 'macroscope' as well as with a microscope." I think marine ecologists and coastal managers have been learning that lesson, and our growing awareness is leading us toward an appreciation for what we often call regional research and monitoring.

The doorbell story suggests a definition of "regional" that may be more useful than that given by the dictionary: a region is the next larger-scale system with influence on our own local field of study. For example, if we are primarily interested in the biogeochemistry and trophic dynamics of a salt marsh on the Potomac River estuary, we will also have to study the Potomac River and the uplands behind the marsh. If we want to understand the Potomac River, however, we must include its watershed and Chesapeake Bay in our study. Thus, from the perspective of the staff of a fictional Potomac River Estuary Marine Laboratory, a study encompassing all of Chesapeake Bay would be thought of as regional marine research. On the other hand, scientists at the several existing laboratories studying Chesapeake Bay recognize the importance of processes in the entire bay watershed, its airshed, and the coastal ocean that influence the behavior of the bay, and they are therefore drawn to regional research on a much larger scale.

The important point is that regional research is not simply large-scale research, and the argument for doing regional research is not that we have learned what there is to know about smaller systems and can therefore move on to larger ones. Rather, the concept of 'region' implies an awareness of, and an interest in, functional linkages among systems. Regional research and monitoring help us understand how events and processes at higher levels of organization, over larger spaces and longer times, influence local ecosystems. Moreover, once we have quantified the influence of these larger-scale processes and events, we will be in a better position to make useful predictions about the future state of our local ecosystem of primary concern.

Let me give a specific example from my own local marine ecosystem, Narragansett Bay. The monthly mean precipitation falling on the Narragansett Bay drainage basin is, on average, remarkably constant over an annual cycle (Fig. 2A). The monthly mean flux of fresh water into the bay, however, varies during the year by a factor of about seven (Fig. 2B). Because there is a close inverse relationship between the flow of fresh water into Narra-

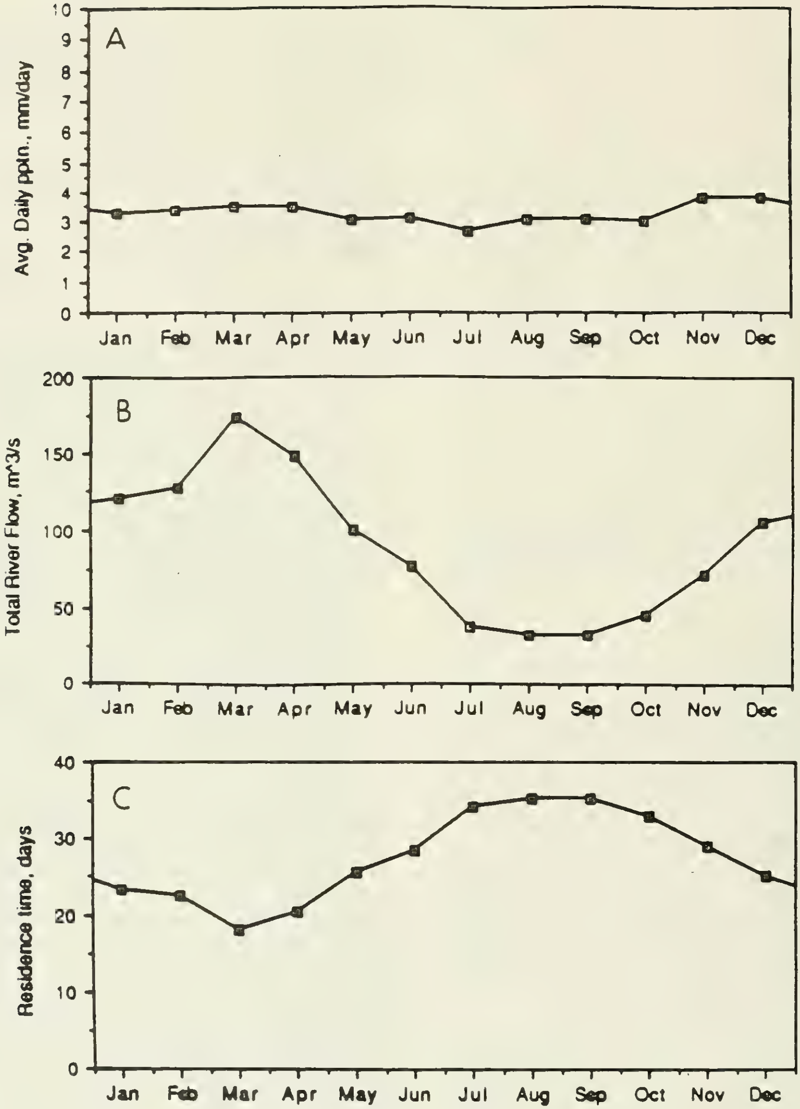

Figure 2. (A) Monthly means of the daily precipitation in the Narragansett Bay drainage basin, averaged over the years 1964 to 1987, inclusive. (B) Average monthly values for the estimated total flow of river water into Narragansett Bay from the watershed, not including the flow of sewage. (C) Mean residence time of the water in Narragansett Bay, calculated from the average total fresh water input and the relationship between fresh water input and the residence time derived by Pilson (1985). (All from Pilson, 1989.)

gansett Bay and the mean residence time of water in the bay (Pilson, 1985; Officer and Kester, 1991), the residence time of the water in the bay varies from about 15 to 35 days during an average year (Fig. 2C). Because the difference between rainfall and runoff is due largely to evapotranspiration by the terrestrial vegetation, it is clear that trees (and land use practices) must play a significant role in influencing the physics of Narragansett Bay. Consequently, the terrestrial vegetation must also influence the biogeochemistry of nitrogen (N) in the bay and the net flux of anthropogenic nitrogen and phosphorus (P) through Narragansett Bay to the coastal ocean. These links come about because denitrification rates in estuaries and the export of total N and P from estuaries to the coastal ocean are largely a function of the mean residence time of the water that flows through them (Fig. 3). Because nitrogen is the major nutrient limiting primary production in Narragansett Bay (Oviatt *et al.*, 1995), we have come full circle with biology on land (people and trees) influencing fresh water hydrography, and thus estuarine physics, and thus estuarine chemistry, and thus estuarine biology.

I realize that my definition does not necessarily include some of the kinds of coastal research that others may think of as regional; *e.g.*, comparisons of chemical concentrations or organismal abundances in a number of estuaries. Comparative studies are important and can help us to develop useful empirical theories, but unless they explicitly include the influences of larger scale processes on the behaviors being compared, I do not think we should consider them as regional research. Furthermore, regional research is not necessarily needed to study a phenomenon simply because it occurs widely. Thus, nutrient enrichment is common in estuaries of the northeast, but we may learn a great deal about how estuaries respond to fertilization by studying only a small sample of them. In contrast, some phenomena can only be perceived as problems, and studied appropriately, on a regional scale. Examples include wetland fragmentation and loss, shoreline erosion, and depletion of migratory fish and wildlife. All of these involve important couplings between local and larger-scale systems.

Some Obstacles to Regional Marine Research

If we accept that regional-scale efforts will expand the size and complexity of coastal marine ecology (and I consider ecology to be the study of ecosystems, not just organisms), then such efforts are also likely to force coastal scientists to interact more with each other, and perhaps to work in teams as suggested by the "food for thought" quotation included in the program for this meeting:

> Ecologists will have to learn to curb some of their traditional individuality, to learn how to work in large teams harmoniously and effectively. . . . What is needed is nothing less than a new psychology or a new sociology for ecologists.
>
> Stanley Auerbach, 1968,
> Congressional Testimony

The requirement for team research is seen as an obstacle by many. The common impression among coastal researchers and research administrators alike seems to be that the scientists who study estuaries and near coastal systems are a parochial, territorial, and even cantankerous lot who cannot or will not work together. We often compare ourselves unfavorably with our blue-water colleagues who seem to work so well together on shared "research platforms" at sea.

I have long been suspicious of this theory because I work at a predominately blue-water institution and my open-ocean colleagues are at least as cantankerous as the coastal scientists I know. To test the theory, I counted the numbers of authors of individual papers appearing in recent regular issues of the journals *Estuaries, Continental Shelf Research,* and *Deep Sea Research.* The theory of "coastal cantankerousness" has never been formally elaborated, but it should reasonably predict that estuarine scientists publish a higher proportion of single author papers than the more congenial teams of deep-water scientists. The results are not consistent with that prediction, but papers involving collaboration of three or more authors seem to be more common in *Continental Shelf Research* and *Deep Sea Research* (Table I). Further, estuarine scientists are much more likely to collaborate only with researchers at their own institution than are those who work on continental shelves or in the deep sea (Table I).

There are several possible reasons for these observations. They may indicate that coastal scientists really are territorial and parochial in their outlook. On the other hand, the tendency to work with colleagues from one's own institution may simply reflect the abundance of scientists interested in estuarine and nearshore work at coastal laboratories. Researchers committed to shelf and open ocean studies are rarer and thus may be driven outside of their parent institution to assemble a team with the skills required to tackle the problems of interest to them. Shelf and open ocean researchers have also been more successful in developing large multidisciplinary research programs that have attracted higher funding levels.

As an aside, I think that the prevalence of multi-institutional work on continental shelves compared to that in the deep sea reflects two interesting relationships. First, the physical oceanography of the shelf is more complex and less well understood than that of the deep ocean. As a result, it is more empirical and less easy to treat mathematically. Papers in *Continental Shelf Research* frequently report the results of ambitious measurement programs involving various specialties, whereas theoretical collaborations between a physicist and a mathematician, or between two mathematicians, are more common in *Deep Sea Research*. Second, my cursory review of the journals suggested that the scale of continental shelf research is such that remotely sensed data from satellites are particularly helpful in resolving physical and biological phenomena. Thus, specialists in analyzing remotely sensed physical (usually temperature) and biological (surface water color) data are often added to teams of seagoing physical, biological, and chemical oceanographers. A numerical modeler is also a common member of the team. Assembling such a group of specialists apparently requires the intellectual resources of a number of institutions.

Table I

Authors and institutional affiliations for regular articles published in Estuaries[1], *Continental Shelf Research*[2], *and Deep Sea Research*[3]

	Frequency (%) of articles with various numbers of authors				
	1	2	3	4	5 or more
Estuaries (n = 121)	15	47	16	11	11
Cont. Shelf Research (n = 123)	12	42	24	15	7
Deep Sea Research (n = 165)	16	34	23	15	12
	Frequency (%) of articles from authors at various numbers of institutions				
	1	2	3	4	5 or more
Estuaries (n = 121)	70	20	8	2	0
Cont. Shelf Research (n = 123)	19	40	23	12	6
Deep Sea Research (n = 165)	52	33	9	4	2

[1] June 1993 (vol. 16, no. 2) through June 1995 (vol. 18, no. 2).
[2] Jan. 1993 (vol. 13, no. 1) through Dec. 1994 (vol. 14, no. 15).
[3] Jan. 1993 (vol. 40, no. 1) through June 1994 (vol. 41, no. 5/6).

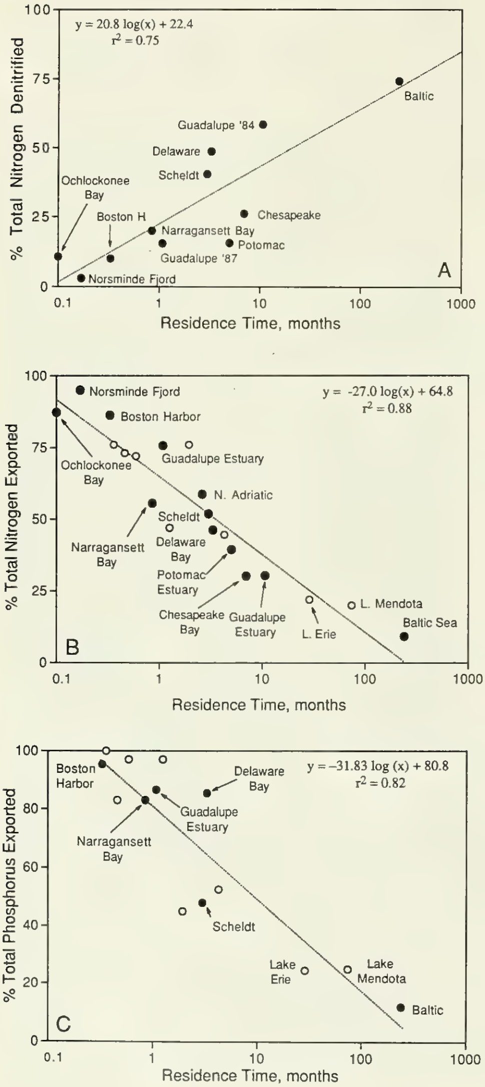

Figure 3. (A) The fraction of total nitrogen input from land and atmosphere that is denitrified in various estuarine systems as a function of mean water residence time. The estimate for Delaware Bay is almost certainly too high because it is based on summer measurements only. Anoxic bottom waters over parts of Chesapeake Bay and the Potomac estuary may reduce the fraction of total N input that is denitrified. (B) The percentage of total nitrogen input from land and atmosphere that is exported from a sample of estuaries and lakes as a function of mean water residence time in the system. The regression line is for the combined data set. Separate regressions for estuarine and lake data were virtually identical. (C) The percentage of total terrestrial phosphorus that is exported from a sample of estuaries and lakes as a function of mean fresh water replacement time in the system. Regression line includes lakes and estuaries but excludes the Guadalupe in a low flow (long residence time) year. (From Nixon *et al.*, in press.)

As a final note on collaboration among coastal scientists and laboratories, I quote from a 1983 National Research Council (NRC) report on Fundamental Research on Estuaries:

> The primary focus of future research in estuaries should be on interdisciplinary relationships. . . . The panel is satisfied that the scientific community will respond with competence and exceptional achievement if funding becomes

> available. Team study, the use of consortia, ad hoc linkages, and other cohesive procedures have frequently been effective in other areas of earth science . . . , and they can be developed and used for estuarine studies.

We are able to test this 12-year-old prediction, at least in part, because of the implementation of the Land-Margin Ecosystem Research Program (LMER) by the National Science Foundation. Data summarized in Table II clearly show that coastal scientists from several institutions can come together in relatively large numbers if the level of support makes it possible, and if agency review procedures and priorities encourage them to do so. Unfortunately, it is still too early to know whether the results of all this collaboration will prove to be an "exceptional achievement." The earlier experiences of terrestrial ecologists and limnologists in the International Biological Program showed clearly that team research does not necessarily produce profound results. As Bruce Peterson (1993) noted in a recent essay on collaborative research:

> . . . it is probably true that even group creativity will asymptote or decline as very large projects can become inefficient and hinder scientific achievement. The maintenance costs of the largest of the collaborative research efforts probably exceed the funds going to research. At the largest scales, little original or creative work can be attempted as the goal requires strong top-down direction and control to address the big question.

If I had been preparing these comments ten years ago, I might have been tempted to emphasize, as important obstacles to regional coastal research, the lack of sophisticated instruments for acquiring data in estuaries and other shallow water areas, and the limitations of computer systems for storing, processing, and sharing data. Fortunately, both of these areas have progressed markedly, and the remaining challenges are the subject of papers by others. I therefore move on to what I think is the most serious obstacle to productive regional research: the danger that we as scientists and managers will embark on regional programs with the attitude that they should be carried out with the same concepts and methods as before, but on a larger scale. If we simply declare victory in the estuaries and move on to watersheds and continental shelves, we will have missed what may be our last chance to make a fundamental change in the way we have been doing environmental research. It will not do to measure more things, more often, in more places.

Table II

Numbers of investigators and institutions engaged in NSF-LMER projects[1]

Site	Investigators	Institutions
Chesapeake Bay	18	1
Columbia River	16	3
Georgia Rivers	12	2
Plum Island Sound	11	3
Tomales Bay	9	5
Waquoit Bay	19	6

[1] *LMER Personnel Directory, 1995.* Researchers based at different campuses of the same university were considered to belong to one institution.

Prediction and Explanation in Ecology

> . . . much of ecology is confused in its goals, uncertain of its strengths, and inconsistent in its terminology . . . ecologists rarely try to make predictions, believing that some special property of their systems makes the common model for building and testing theories inoperable.
>
> Rigler and Peters (1995)

It is characteristic of complex systems that their past behavior is easy to explain while their future state is very difficult to predict, and the coastal marine environment is, without question, a mosaic of very complex systems. Unfortunately, coastal ecologists have developed the art of explaining the behavior of these systems and all too often have neglected the science of predicting their behavior. Because we confuse understanding with explanation, we fill our scientific literature with "stories" about why algal blooms occurred when and where they did, or why there were more worms or fish or birds in the bay one year than in another. The review process tests these stories to see if they "make sense"—that is, if they are consistent with the conventional wisdom about how nature "works." Because there are so many variables, so many fluxes, forcing functions, and feedback loops, a large number of explanations is always possible and even reasonable. Moreover, humans are notoriously good at seeing patterns that reinforce their beliefs while missing or ignoring those that do not. Even if the measurements that we make don't quite fit our story, we can always argue that we didn't measure everything (another predator may have been present, etc.), that we only measured for a short time and thus hit a transient, that we measured in a patchy environment and thus didn't take enough measurements, or that the time or place of our study was in some way "atypical." One of the most common endings to our stories is to remind the reader that the phenomenon under study is complex and that the blooms or worms or fish probably resulted from the interactions of several factors, and so on endlessly.

Our stories are not improved by their surfeit of fuzzy words and concepts that lack any operational definitions. Even the 1994 NRC report on Priorities for Coastal Ecosystem Science is full of references to "ecosystem health," "ecosystem integrity," "ecosystem degradation," "habitat quality," "ecological restoration and rehabilitation," "key parameters," "key interactions," "ecosystem structure," "verified models," etc. To my knowledge, none of these terms has any generally accepted operational meaning. What shall I measure to know if the integrity of Narragansett Bay is increasing or decreasing? If we really knew what key parameters or processes were, we wouldn't have so much trouble deciding what to monitor. I acknowledge that at least some of these terms may have real meaning to at least some of the people who use them, but as a community of scholars, we do not set a rigorous enough standard on our language. After 20 years of reading and writing about coastal marine eutrophication (another big topic in the 1994 NRC Coastal Ecosystem Science report), I finally made an effort to define what I had been working on (Nixon, 1995):

> Eutrophication (noun)—an increase in the rate of supply of organic matter to an ecosystem.

Such a definition should make future discussions of eutrophication clearer and more focused, because it is operational—it says what to measure. We may have productive debate about how to measure carbon input, about how great a change is required for statistical significance, etc., but we can at least stop confusing cause and effect or process and state.

A major consequence of our storytelling and elusive terminology is that we do not make much progress in doing what most people (including the funding agencies) want us to do—make predictions about the future state of coastal resources and ecosystems. Marine ecology has been presenting itself as a quantitative science for about 100 years, yet we almost always fail at answering the "what if" questions. The late F. H. Rigler and R. H. Peters have written clearly and compellingly about this problem in *Science and Limnology,* the latest volume in the Excellence in Ecology series from the Ecology Institute:

> I contend that the body of ecology is infected by a strange disease. The infection is not new, but its extent was unappreciated until our troubles were brought into the open by a set of unusual circumstances. Just as a heavy snowstorm reveals a blockage in the coronary artery of the hapless shoveller, the environmental crisis has revealed the unhappy state of ecology. The disease of ecology is that it is a science that lacks theories it thought it had. When we are asked to predict the impact of a new dam or of an oil spill, or of chronic chemical contamination, we cannot do it. . . .
>
> We may want to do more than predict. For example, we may want to explain or to understand nature. We may claim that we can do more, but those claims only demonstrate our naivety. Science cannot do more. The only way that we can demonstrate our understanding of nature is with predictive power; all else is opinion and posture.
>
> R. H. Peters
> *Science and Limnology* (1995)

By retelling essentially the same stories for different bays and estuaries (albeit with interesting local variations), we easily suffer the delusion that our common and recurring explanations reflect understanding. But papers that make a falsifiable prediction are as rare in marine ecology as they are in limnology. Even simple and falsifiable empirical theories, such as those shown in Figure 3, are very unusual in coastal ecology. Unfortunately, I suspect that many coastal ecologists will dismiss such simple empirical relationships as trivial compared with elaborate mechanistic explanations of plankton blooms or fishery declines. But the simple empirical theories (stated in this case as regressions) are easily tested, and they are falsifiable. As long as they "work" (that is, remain reliable), they are useful predictive tools for managers, and they serve as solid starting places from which we can proceed to develop more sophisticated theories to explain the simple ones. If a system does not follow a regression such as one of those in Figure 3, then we must ask specific questions about it and try to develop a new theory. Thus, even a simple, clearly stated theory leads toward self-correction and toward progress in understanding—that is, toward the development of richer theories that predict more about the future state of the system and how different parts of it will respond to various kinds of change.

The NRC (1994) report on Priorities for Coastal Ecosystem Science did recognize the importance of prediction, but seemed to relegate it to the special province of modeling:

> A priority for . . . ecosystem modeling research is "to establish improved predictive capabilities for coastal ocean systems that link physical processes, biogeochemical cycles, and the interactions of living marine resources."

The quotation within the quotation is from a 1993 report from the federal agencies Subcommittee on U. S. Coastal Ocean Science (SUSCOS). The need for a predictive total ecosystem (or world) model cannot be gainsaid, but as a priority for research, it doesn't help much. Unfortunately, this is a recurring problem in the NRC (1994) report, as well as virtually all the other recent coastal science planning documents I have reviewed. Not only do we in the coastal community focus on explanations rather than predictions and use a lot of undefined terms, we also set unrealistic and fuzzy goals. Again and again, the goal of the various activities called for in the NRC (1994) report is "to increase our understanding." Because virtually any measurement or observation can be justified as leading toward an increase in our "understanding," this is not a helpful goal. As Rigler and Peters (1995) argue so well, a major contribution of 20th century philosophy has been the demonstration that there is no absolute truth. No matter how much money or time, or how many research assistants and buoys and boats and meters I have, I will never come to the point when I can stand back and say, "At last, I have it! I understand Narragansett Bay."

What is possible, however, is to develop ever more powerful and encompassing theories that will enable us to predict, at least under some circumstances, the probable future state of coastal marine ecosystems and various parts and processes of those systems. If we insist that the theories be falsifiable statements with operationally defined terms, we can continuously test the theories and revise or reject them when they fail. We can begin to answer at least some of the "what if" questions. We can demonstrate that our research is worth supporting and that our science is worth teaching. We can make progress. We can do science rather than storytelling.

A Role for NAML

The theme of the workshop that precipitated this essay is "the role of marine laboratories in implementing the nation's emerging priorities for research and monitoring in the coastal zone." Perhaps some priorities are emerging, but I don't think they have yet emerged. An important role for the scientists at NAML institutions is to keep working with federal and state agencies to express priorities in clear, operationally defined terms. We should struggle hard to begin to cast those priorities in the form of testable or falsifiable predictions from new and existing theory. This will not be easy. We will need to stop talking about increasing our understanding and start asking ourselves more specific and difficult questions. What do we really want coastal marine science to be able to do five years and ten years from now? Which of those hopes appear realistic given the resources that will probably be available? Can we express our present "understanding" of the interactions between local- and regional-scale ecology in testable formulations or theories?

I don't know what the role of NAML as a group of institutions should be. Surely a major share of the nation's intellectual infrastructure for coastal research resides within the influence of NAML institutions. For this reason alone, NAML should accept some responsibility for a self-examination of the way we have been doing estuarine and coastal research.

Perhaps the criticisms that Rigler and Peters (1995) directed so clearly and provocatively at their fellow limnologists do not apply to marine ecologists. Unfortunately, I think that a large part of their argument applies to our field as well. We have, of course, learned many new things about coastal environments during the past 50 years—many of those discoveries are intellectually pleasing; some are important to practical concerns. But by and large we do not organize and express our knowledge in ways that stimulate intuitive leaps toward the formulation of falsifiable theories.

Because intuition and theory are definitely not the province of institutions or associations, but are instead the beautiful and still mysterious product of the individual human mind, we cannot ask NAML (or NSF or EPA or NOAA) to develop theories. Because successful institutions have an interest in maintaining the *status quo,* I am not even sure we can expect NAML to help much with a critical examination of the way we do business. On the other hand, the resources devoted to coastal marine research will certainly not continue to grow as they have in the past. In real terms, they may decline markedly. We cannot expect the taxpayers to continue to support work that does not lead to useful knowledge. Environmental and resource regulations based on fuzzy ideas and nice stories will not endure or serve to protect the environment in the long run. If coastal marine scientists and the laboratories in which they work are going to continue to enjoy public support, they will have to make themselves understandable, credible, and useful to the public. If we are going to embark on more expensive and visible regional research programs, then a careful and critical look at the state of coastal marine science becomes even more important. NAML should play a lead role in that examination.

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Interdisciplinary Research at the Nation's Coastal Laboratories

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Abstract. The nation's coastal (*i.e.*, marine and Great Lakes) laboratories and programs of aquatic research face serious challenges for survival in the decades to come. Although research in these institutions is strongly interdisciplinary, this characteristic and its effect are not widely recognized. Examples of successful interdisciplinary research are many, encompassing a wide range of scales of operation, from the varied technical approaches taken in individual laboratories to multi-laboratory, multi-institutional and multi-platform programs of very large scale. Our survey of interdisciplinary research in the nation's marine and coastal laboratories leads to three conclusions: (1) The trend toward collaboration in marine research is scientist-driven, rapid, and inexorable. It is driven by problems of increasing complexity, and the recognition that multiple approaches are synergistic and powerful, producing answers where single approaches cannot. (2) This trend is rapidly increasing the diversity of expertise and technical competence among coastal laboratories. (3) And the research community and its support agencies must therefore find new ways of representing this rapidly increasing diversity within the country's marine laboratories and their programs. Given past successes, we may be tempted to push forward with large multidisciplinary programs, but we should not neglect the funding required by innovative individual scientists. The marine and aquatic research community and its funding agencies face a major challenge in the years ahead: they must develop a publicly recognizable, national agenda for marine and coastal research that adequately represents the broad spectrum of interdisciplinary research entailed, while ensuring that nonconventional work and innovative "individual" science be effectively funded. Such a balanced approach is most likely to effect a sustained economic development of the nation's coastal zone.

Introduction

Few institutions in the world have, as a matter of tradition, prized interdisciplinary research so greatly, or carried it out so effectively as marine and aquatic laboratories (National Association of Marine Laboratories, 1995). One of the tenets of aquatic field research has been that interdisciplinary approaches are the methods of choice, if not necessity. This has not, of course, meant that more focused research has suffered as a result—quite to the contrary. Marine organisms have been exploited as model systems for pioneering studies of cellular immunity, fertilization, development, nerve action, memory and circadian rhythms, to name a few areas. But as discussed below, even these elegant laboratory studies of fundamental biomedical significance often require skills and approaches far beyond those usually needed for traditional disciplinary work. Almost without exception, however, when the move is made from the laboratory to the field, or from the organism to the environment, multiple disciplines become the norm. Indeed, without interdisciplinary approaches, the major successes enjoyed by marine laboratories over the past few decades would have been unimaginable. For example, the very act of sampling aquatic and sedimentary environments in an understandable and predictable way has required that the skills of ocean engineers and physical oceanographers be melded with those of the biologists or marine chemists needing the samples. One might cite as

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an example of this wedding of disciplines, the many contributions of John Isaacs of the Scripps Institution of Oceanography, whose engineering insights impacted nearly every biologist he contacted during his many years of work. In fact, the physical organization of early marine laboratories almost forced a flow ideas: single buildings often housed chemists, geologists, engineers, and biologists, and their students, who were therefore exposed to different avenues of thought, both through training and through mutual interactions.

Accompanying the obvious need for judiciously placed and timed sample acquisition and measurement was the early realization that the biological factors being studied were intimately connected with chemical and physical factors. This realization prompted the organization of multidisciplinary research units like the Food Chain Research Group at the Scripps Institution of Oceanography, and the Ecosystems Center at the Marine Biological Laboratory, where chemists, biologists, scientists using remote sensors, and others could share ideas about and approaches to, the study of natural aquatic systems. The thinking of such groups has led to an appreciation of scale (both spatial and temporal), without which biological results cannot be interpreted. Some of the first global climate studies were done in association with major interdisciplinary groups of scientists from, and coordinated by, marine laboratories (Houghton *et al.,* 1990).

The tradition of interdisciplinary education and public outreach has also been strong (*cf.,* Milliman, 1996). For many years, students at some marine laboratories have been required to assimilate a curriculum containing physical, chemical, and biological oceanography as part of their basic training. Indeed, many who were trained within a single traditional discipline have slowly developed the knowledge and skills of interdisciplinary thought, if for no other reason than for the need to converse with their own students—a kind of intellectual self defense. A good example is the increasing use of the tools of biotechnology in many areas of marine research—facilitated by training programs for students, postdoctoral Fellows and established investigators (Joint Oceanographic Institutions, 1990; National Research Council, 1994).

We have surveyed the nation's coastal laboratories and have found a broad spectrum of interdisciplinary research. We sought for the common characteristics and needs of these programs. Below we highlight just a few examples representing a gradient of subjects, from the global to the nano-scale. We suggest, finally, that most programs of marine research and scientist-training are similar in their interdisciplinary philosophy and approaches.

Plate Tectonics, Hydrothermal Vents, and Vent Communities

In the 1950s the general shape of the ocean floor, and especially the existence of the mid-ocean ridge system, became clearer through the work of physical oceanographers and geologists. Of great importance to this were data showing magnetic anomalies, which led ultimately to the discovery of sea-floor spreading at the mid-ocean ridges (Vine and Matthews, 1963). In 1968, researchers began using the research ship *Glomar Challenger* to aid in the drilling of the deep sea, adding the discipline of paleo-oceanography to the scene. Finally, in the mid 1970s, with the advent of submersible technology, it became possible to study precisely very small areas of the ocean floor (Ballard and Van Andel, 1977), allowing biologists to participate directly in the study of the ridge environments for the first time, even at depths of 3000 meters and more. The association of the ridge system, which consists of a branching 75,000-km system that encircles the globe, is often, but not always, associated with volcanic activity. In areas of rapid spreading, volcanic activity is likely, and hydrothermal vents, many of them rather spectacular, occur commonly. The effects of these vent systems on both oceanic chemistry (*i.e.,* it is estimated that the volume of the ocean flows through these vent systems in ten million years, changing the chemistry of the seawater as it is heated to temperatures of 300–400°C), and deep sea biology (*e.g.,* large communities of organisms exist in the deep sea because of the chemical energy supplied by thermal reduction of seawater sulfate), are truly dramatic, as discussed by many authors (see Childress, 1988).

Perhaps nowhere in marine science have students been exposed to a more interdisciplinary project than in the area of hydrothermal vent research. The vents were discovered through physical (temperature anomaly) detection methods, and this was followed by extensive chemical research on high temperature fluids and biological research on the fascinating vent organisms and communities. The discovery of these communities in the late 1970s led to a literal invasion, a kind of scientific "gold rush" to the vents, where the skills of physical, geological, chemical, and biological oceanographers were combined, perhaps as never before. Furthermore, the widespread occurrence of symbiosis and the importance of bacteria in this system attracted biologists studying virtually the entire food chain: again, this represented an almost unprecedented cooperation within the broad discipline of biology.

When examining a seemingly simple diagram of the vent ecosystem, it is hard not to marvel at what has been learned by combining the expertise from multiple disciplines. Such pictures have arisen from a point of virtually zero knowledge in only a few years, and they are especially remarkable because these environments are often at depths of 3–4,000 meters, requiring great technical expertise and sophisticated sampling equipment, like the submersible ALVIN or remotely operated vehicles (ROVs), for their study. Such equipment and expertise are available largely through the development of submersibles and ROV technology at marine laboratories like the Harbor Branch Oceanographic Institution, the Woods Hole Oceanographic Institution, and others.

As the story of the vents has unfolded, the impact has become even more interdisciplinary and global—we know now that the network of vents stretches around the world, perhaps 75,000 km or more in extent, and has a massive impact on the chemistry of the ocean and the evolution of the planet; such a system cannot be appreciated without global modelers and plate tectonics experts.

Symbiosis and Marine Biology

At the other end of the size scale, the studies of the symbiotic bacteria at the base of the food chain in vent environments have highlighted a new kind of thinking about the possible importance of chemoautotrophy in general, and about the significance of similar lithotrophic symbioses in many other environments on the surface of the planet. They have also led to the discovery of some unique biochemistry involving specialized proteins for the binding and transport of sulfide—proteins and processes never imagined before the detailed studies by biochemists (see Childress, 1988). The symbionts that live with a variety of the different vent invertebrates (clams, pogonophoran worms, etc.) have until today resisted culturing, but have been identified and studied with modern methods of molecular phylogeny employed by molecular biologists (see Cavanaugh, 1994). Another impact of these studies was to suggest that many other sulfide-rich, but non-thermal environments might harbor symbiotic organisms similar to those so obvious in the vent systems. This has proven to be the case, as shown by many workers and discussed by Cavanaugh (1994).

These discoveries have expanded the interdisciplinary work on the vents still further, as oceanographers, physiologists, microbiologists, biochemists, and molecular geneticists work together to understand the evolution and operation of the hydrothermal vent communities that obtain their metabolic energy from the earth's core, rather than from the sun. Collaborations with industrial biotechnologists also have entered this picture. Private firms are now developing and marketing enzymes from the hydrothermal vent bacteria for use as catalysts that work under extreme heats and pressures.

While the vent studies stand out as perhaps the most extreme example of interdisciplinary research in recent years, they are in no way unique; almost all other studies involving field work are to one extent or another similar in approach, if not extent. One may cite, for example, the recent investigations of an entirely different symbiotic system, that of the luminous squid *Euprymna scolopes*, and the luminous bacteria that supply light for the squid. These fascinating squids harbor luminous bacteria in specialized light organs; the light produced by the bacteria enables the host to escape from predators. Because the light is produced continuously, the squid has evolved a series of filters, shutters, and regulatory mechanisms. The bacteria, when outside the host squid, are non-luminous, so molecular probe methods must be used to identify them (Lee and Ruby, 1992). Newly hatched squids lack luminous bacteria, and if raised in the absence of bacteria, they fail to develop the luminous organ (McFall-Ngai and Ruby, 1991). Thus, the entire story, as it unfolds, involves the skills of molecular biologists, microbiologists, cell biologists, visual physiologists, functional morphologists, and developmental biologists—laboratories full of students with different specialties, thinking about solutions to the same problem. To understand the impact of the squid on the distribution of the bacteria, physical oceanographers and modelers will eventually be included. And if the role of the symbiosis in the evolution of the animal is to be understood, the group of scientific participants will need to be enlarged even further.

Zooplankton Behavior

Another example of quite a different ilk is that of zooplankton behavior (feeding, predator avoidance, mating, etc.). The feeding and behavior of small zooplankters in laboratory culture systems that were often isolated from the physical and chemical regimes in the ocean have been studied by biologists for many years. Recent interdisciplinary approaches, involving underwater laser optics and high speed data acquisition systems, have led to new insights about feeding mechanisms and swimming behavior and the role of physical forces such as turbulence and gravity on these processes. When these approaches were combined with computer assisted mathematical analysis of the data, understanding about how small zooplankters sense and respond to the presence of food and predators increased markedly (Strickler, 1985). This work has led to the development of new optical approaches (Strickler and Hwang, 1996), and the establishment of interdisciplinary groups that include engineers for equipment development, zooplankton biologists, statisticians, and physicists studying the dynamics of water movement (Squires and Yamazaki, 1996; Yen, 1996).

Recruitment, Fisheries, and Aquaculture

The recruitment of economically important resource species has emerged as a research focus of national importance, as our major fisheries, and those of the rest of the world, are collapsing. But the complex processes that govern recruitment are inexplicable without the combined efforts of oceanographers, ecologists, larval biologists, and remote sensing specialists. Once thought to be completely random, the settlement and metamorphosis of the planktonic larvae from a wide variety of benthic marine invertebrates (including many valuable resource species), in the laboratory and in the natural environment, are controlled by larval recognition of exogenous chemical signals (reviewed in Scheltema, 1974; Chia, 1978; Hadfield, 1978, 1986; Crisp, 1984; Rittschof and Bonaventura, 1986; Morse, 1990; Pawlik, 1992; see also: Highsmith, 1982; Hadfield and Scheuer, 1985; Jensen and Morse, 1990). In some species, this sensory recognition is required to activate resumption of the genetically programmed developmental processes that had been arrested during the period of larval dispersion (Morse, 1990), while in others, negative cues may deter settlement and metamorphosis on inappropriate substrata (Johnson and Strathmann, 1989; Woodin, 1991; Holmstrom *et al.,* 1992). Interdisciplinary collaborations between recruitment ecologists, larval biologists, oceanographers, hydrodynamicists, biochemists, and molecular biologists are now starting to reveal how larval recognition of these chemical cues from the environment interacts with hydrodynamic and other factors to control settlement from the plankton, metamorphosis, and recruitment (*e.g.,* Wethey, 1986; Jensen and Morse, 1990; Pawlik *et al.,* 1991; Mullineaux and Butman, 1991; Butman and Grassle, 1992; Grassle and Butman, 1992; Pawlik and Butman, 1993). Thus, for example, Zimmer-Faust and his colleagues have shown that oyster larvae can follow plumes of soluble peptides and thus locate settlement sites near conspecifics (Sammarco and Heron, 1994; Turner *et al.,* 1994; Zimmer-Faust and Tamburri, 1994).

The tightest control of recruitment by substratum-specific settlement cues in the natural environment has been found in those species where the larval requirement for an exogenous inducer is of the highest stringency and specificity (Morse, 1990). In these species, the capacity to delay metamorphosis in the absence of a required inducer seems to enhance both the dispersal and substratum-specificity of the final distribution of the recruits. The complexity of the larval chemosensory mechanisms controlling metamorphosis in response to both substratum-associated and water-borne chemical cues (Rittschof *et al.,* 1986; Baxter and Morse, 1987, 1992; Coon and Bonar, 1987; Fitt *et al.,* 1987; Bonar *et al.,* 1990; Maki *et al.,* 1990; Wodicka and Morse, 1991; Morse, 1993), and the sensitivity of these systems to regulation at multiple levels, may provide the larvae of some species with rather remarkable abilities for fine-scale discrimination of chemical features of the environment that simply were not anticipated previously.

In collaborations between larval biologists, ecologists, oceanographers and remote sensing specialists, this information has been combined with data defining the important roles of hydrodynamic, topographic, and geographic features that control larval dispersal (*e.g.,* Frith *et al.,* 1986; Black, 1988, 1993; Roughgarden *et al.,* 1988; Sammarco and Andrews, 1988), and used to predict spatial and temporal patterns of recruitment. These collaborative efforts are producing important new methods for use in fisheries management decisions (*e.g.,* Tegner and Dayton, 1991; Barry and Tegner, 1990). Although most of these studies have been conducted with larvae and recruits of benthic invertebrates, the role of niche-specific requirements that may control the recruitment of fish larvae are starting to gain attention. In the GLOBEC (Global Ocean Ecosystem) and other multi-institutional efforts, collaborations between fisheries biologists, oceanographers, remote-sensing specialists, and molecular and population geneticists are charting the oceanographic and climatological determinants that control the recruitment of the world's major fin-fish resources (National Research Council, 1991, 1994). Molecular and population geneticists are helping to refine these models, with DNA-fingerprinting and other genetic data helping to resolve the populations of origin, and paths of dispersion and migration (Joint Oceanographic Institutions, 1990; Bermingham *et al.,* 1991). The signals and molecular mechanisms controlling larval metamorphosis in resource species of invertebrates also have found wide application in commercial aquaculture (Morse, 1992).

High-Performance Marine Biomaterials

To help meet the needs for a new generation of advanced, high-performance composite materials, polymers, coatings, and medical devices, materials scientists and engineers are collaborating with marine scientists to develop methods for biomimetic synthesis of a wide diversity of specialized materials modeled on those produced by marine organisms. These range from the mineralized nanocomposites and nanoporous materials of molluscan shells, coral skeletons and diatoms (Eisner, 1991; Heuer *et al.,* 1992; Mann, 1992, 1993; Pennisi, 1992; Mann *et al.,* 1994; Walsh *et al.,* 1994) and the remarkably strong underwater adhesives and corrosion-resistant coatings produced by marine mussels and other invertebrates (Waite, 1990, 1991; Waite *et al.,* 1992; Hansen *et al.,* 1995), to the rich store of biopolymers such as chitin, agar, and carrageenan (Office of Technology Assessment, 1993).

The marine biomineralized composites are especially promising in this regard, but they present special challenges that require non-traditional collaborations. Many of these materials are novel ceramic-like composites of highly oriented mineralized microcrystalline domains and protein-based biopolymers, with anisotropy, biocompatibility and high strength:weight ratios far exceeding those of the minerals themselves. Thus, they provide attractive models that can be used to guide the design of new synthetic composites with unusual mechanical, optical, magnetic, electronic, catalytic and photo-catalytic properties. Such materials might be used to fabricate new microelectronic, optoelectronic and catalytic devices, and smart medical and dental implants. These durable composites might also be used in construction, especially in moisture-shields.

The molluscan shell, for example, is a microlaminate composite with ceramic-like consistency that exhibits remarkable strength and nanoscale regularity. Consisting of calcium carbonate crystals and proteins, its fracture-resistance is about 3000-fold greater than that of the mineral component alone. The strength and organization of the material are determined largely by the proteins, which constitute only 2% of the composite by weight, but control the nucleation, orientation and packing of the microcrystalline domains. Materials scientists interested in learning how the natural composite is made, and aiming to duplicate the process, hope to control mineral crystal orientation and packing by templating crystal nucleation and growth on ordered arrays of these proteins or their synthetic analogs. But there are major barriers to this strategy: The proteins are present in very low amount and are difficult to recover from the mineralized shell; the dynamic mechanisms of crystal-organic nanoscale assembly controlled by these proteins are poorly accessible to observation and experimentation; and the composite materials are produced in nature by growth mechanisms that are baffling to human engineers. New interdisciplinary collaborations between marine biologists, molecular biologists, chemists, physicists, and materials scientists and engineers are needed to solve the formidable technical problems; new model biological systems for analysis must be developed; the proteins must be identified and characterized via their cloned cDNAs; and advanced physical instrumentation must be adapted for the requisite nanoscale analyses. Using the atomic force microscope, x-ray diffraction and Raman microprobe techniques—in conjunction with a new "flat pearl" model system to facilitate analysis (Fritz *et al.,* 1994; Zaremba, 1996)—one such interdisciplinary group recently discovered a new mechanism of nacre growth (Mann *et al.,* 1993), resolved the different proteins responsible, and used these proteins to produce microlaminate composites *in vitro* (Belcher *et al.,* 1996).

Marine Organisms as Models for Biomedical Research

The history of biomedical research is replete with examples of fundamental mechanisms that were first discovered in experiments on marine animals. Significant breakthroughs continue to come from research with these systems, often performed in marine laboratories. This research typically costs far less, and is completed years sooner, than research confined to mammalian systems could be. While the principal researchers are likely now to be molecular or cellular neurobiologists or developmental biologists, they too depend on interdisciplinary teams providing expertise in marine animal production and cultivation, advanced imaging technologies, electronics, hyperbaric engineering, etc.

Highlights of some of the most significant biomedical discoveries made with marine organisms, with three examples of Nobel Prize-winning work, include the discovery of cellular immunity, histocompatibility, and the genetic dissection of self-nonself recognition mechanisms (starfish, ascidians, hydroids); discovery of the mechanisms, receptors, and genes controlling fertilization and early development (sea urchin, starfish, abalone); discovery of cyclins and other molecules controlling cell division (clams, echinoderms); elucidation of the mechanism of synaptic transmission (electric fish); discovery of the action potential and elucidation of the mechanism of axonal transmission (squid); identification of the cellular and molecular mechanisms responsible for neuronal facilitation, learning and memory (marine gastropods); early discoveries of neurosecretion and neurohormones, and elucidation of neuronal and neuroendocrine mechanisms regulating reproduction, cardiac physiology and circadian oscillators (marine crustaceans and gastropods) (National Research Council, 1985).

Reproduction, development, growth, physiology, and disease in these marine animals are controlled by genes, molecules, and mechanisms that are homologous to those in humans. This homology of structure and function—known at the National Institutes of Health as "high connectivity" (National Institutes of Health, 1991)—combined with the unique specializations and ease of culture afforded by marine invertebrates, provides a diversity of specialized systems that are uniquely tractable for experimental analysis of many basic mechanisms. As illustrated in the list of discoveries above, marine molluscs possess specializations that make them uniquely suited for dissection of the molecular and cellular mechanisms of the nervous system. Similarly, breakthrough discoveries in fertilization and development continue to be facilitated by the use of systems such as fish, sea urchins, clams, and abalones, which provide the unique experimental advantages of millions of gametes, embryos, and larvae, available on demand, throughout the year; external (hence fully controllable) fertilization; rapid embryonic development; metamorphosis controlled by exogenous signal molecules; ease of egg-to-egg cultivation; and amenability to molecular and cellular manipulations (National Research Council, 1994). Thus the most detailed information available on the molecular mechanisms of egg-sperm recognition (Foltz and Lennarz, 1993), receptor mediated egg activation at fertilization (Foltz and Abassi, 1994), and transcriptional regulation during early cell differentiation (Davidson, 1994; Zeller *et al.*, 1995) continues to be obtained first in the sea urchin system.

Common Denominators and a Common Focus

Marine laboratories have pioneered the development of interdisciplinary approaches to local, regional, and global studies. These range from regional coastal studies (Nixon, 1996) and expeditions to specific sites (*e.g.*, the Black Sea, Sargasso Sea, Cariaco Trench), to longer-term environmental studies (Feller and Karl, 1996), and ambitious global programs like JGOFS (Joint Global Ocean Flux Studies) aimed at understanding the workings of the global climate system. JGOFS, a long-term interdisciplinary project, comprises investigations from the global level (via the use of satellite monitoring and remote sensing) to the microscale (via fluorescence cell sorting analyses of plankton dynamics) and the use of DNA and RNA molecular probes of organismal metabolism and distributions. Such programs reflect the wide recognition that solutions to large and complex problems can only be obtained through coordinated interdisciplinary efforts of many investigators.

As marine scientists increasingly work with atmospheric scientists, remote sensing specialists, chemists, physicists, geologists, engineers, biotechnologists, and medical researchers, the diversity of approaches and skills in our marine and coastal laboratories will continue to increase rapidly. This is the inevitable result of pursuing the cutting edge of the science in each of these endeavors. Moreover, it is in the national interest that this growing breadth of approach not be constrained within a limited or outmoded view of what a marine laboratory should be doing. The common denominators in this growing diversity include (1) the need to solve pressing problems in complex systems, and the resulting requirements for multiple skills and approaches; (2) the requirement for costly instrumentation or infrastructure; (3) a general lack of understanding, by both the public and Congress, of the importance of the issues under investigation; and (4) the lack of any coherent or compelling national focus—let alone a national priority—for marine or coastal research.

Because the increase in interdisciplinary collaboration is research-driven, rapid and inexorable, and because this will continue to increase the diversity among the nation's coastal and marine laboratories, the challenge to the marine science community and its support agencies is to protect this diversity, while at the same time forging some national identity that can serve as a magnet for recognition, understanding, and support—in much the same way that the National Institutes of Health and National Atmospheric and Space Administration are recognized and supported. Representing the diversity of strengths and expertise among the nation's coastal and marine laboratories under the single banner of "coastal monitoring," for example, would be no more appropriate or effective than representing the accumulated national capital of their research strengths under the flags of "marine ecology," "aquaculture," "global climate," or "drugs from the sea."

The nation's future economy will depend on sustained development in the coastal zone: Most of the population is on the coast; shipping and commerce, tourism, oil, and energy production all are focused here; defense conversion and the continuing national defense are major issues here; traditional resources like fisheries, and new and developing resources like offshore minerals, marine biomedical and biotechnological resources, and aquaculture all are based here; and all of these are controlled by the ocean's engine driving our climate system. We suggest that the nation's coastal research institutions join with their support agencies in developing a unifying association between the research in each of the areas of their special strengths and the sustained economic development of the coastal zone. This is the approach that marine scientists and support agencies have used with conspicuous success in Norway and Japan. We suggest that this approach would benefit the United States as well.

The crisis in research funding and its management, faced by the United States today, highlights the need to address major interdisciplinary problems while ensuring support for innovative investigator-initiated research: No area of marine research illustrates this present crisis more clearly than the imperative for coordinated interdisciplinary research in the area of climatology and the effects of climate change. It is widely recognized that this area requires the sustained and coordinated interdisciplinary collaborative work of oceanographers, atmospheric scientists, meteorologists, hydrologists, climatologists, paleoclimatologists, geologists, systematists, physiologists, ecologists, evolutionary biologists, remote-sensing and information-processing specialists, coastal process engineers, fisheries and resource biologists and managers, modelers and specialists in coastal policy and international trade. Because the marine and coastal laboratories of the United States potentially constitute the world's best "early-warning" network for detection and prediction of the effects of global climate change, focused research and monitoring at these institutions must be a national priority.

The cognizant federal agencies have tried to do all that they can to support this effort wisely, and they have called on the research community for guidance in this effort. But no national-level mechanism has yet been devised to ensure proper review and sufficient funding of such "big ocean science" while at the same time safeguarding support and encouragement for innovative investigator-initiated research across a broad range of disciplines. The Ocean Studies Board of the National Research Council found that (1) the funds available are insufficient to maintain both kinds of science; (2) coordination between the marine-related support programs of the NSF and mission-oriented agencies is vitiated both by insufficient support and lack of priority; and (3) the checkerboard of federal and state, private, public, and academic marine laboratories generates a confused array of competing needs (National Research Council, 1992; Wunsch, 1993; *cf.*, Malone and Nemazie, 1996). Therefore, the challenge faced by the marine science community and its funding agencies is to develop a publicly recognizable national agenda for marine and coastal research. This program should support the broad spectrum of interdisciplinary research necessary for sustained economic development of the nation's coastal zone but must also ensure that nonconventional work and innovative individual science are effectively funded.

The scientific approach to large and complex problems (such as climate change) requires special mechanisms to facilitate the work of large interdisciplinary (and inter-institutional) groups. Electronic linkages will facilitate coordination, shared use of data, and will extend observational baselines (Feller and Karl, 1996). But as concluded by a special workshop panel convened by the National Association of Marine Laboratories (1995), the development of interdisciplinary approaches is best left to the participating scientists and the nation's laboratories on a competitive basis. Disproportionate allocations to "training programs" can no longer be the answer relied upon by the research community and support agencies, because (1) the supply of trained scientists now exceeds the nation's present capacity for employment in research (Bloom, 1995), and (2) training programs do not cover the costs of the research they require to accomplish their training objectives. It is a symptom of the present illness of the country's research support infrastructure that obtaining a "training grant" is in many areas, easier than obtaining funding for the research necessary to train scientists and to keep them employed. Other "top-down" management devices designed to foster interdisciplinary research will also inevitably short-change the nation, as they have proven economically wasteful in the past (*cf.*, Malone and Nemazie, 1996). Moreover, they fail to keep pace with the rate of discovery and with changes in the way research is done.

Chief among our recommendations is that proposals to agencies requesting support for interdisciplinary research be evaluated by special review panels (*cf.* National Association of Marine Laboratories, 1995). While such panels are used routinely by some agencies for the large established programs, they also are needed to prevent the dumping, by the more traditional discipline-based review panels, of proposals for innovative interdisciplinary collaborations in new areas. Identifying large interdisciplinary programs that have not yet been discussed adequately by national policy-makers may also be disadvantageous. When agencies earmark research funds for large programs like GLOBAL CHANGE, GLOBEC, JGOFS, RIDGE, BIODIVERSITY, etc., the pool of funds remaining for support of innovative, individual investigator-initiated work in other areas is generally reduced (*i.e.*, program-specific augmentations rarely surviving intact for multiple years). Funding for innovative work is further constrained by the establishment of a "bandwagon" mentality, which, in some cases, excessively limits the subjects and approaches of investigation (*e.g.*, various "National Aquaculture Plans" of the past, that for reasons not free of the political, excessively limited the range of species on which investigations might be funded).

NSF and ONR, with guidance from the scientific community, have been at the forefront of the effort to balance the competing needs for marine research dollars; but the magnitude of NSF support for ocean science has lagged far behind the growth of total NSF support (National Research Council, 1992; Wunsch, 1993), and the other Federal agencies have been far less effective either in seeking or in following recommendations from the research community. Until the recent establishment of NAML (The U.S. National Association of Marine Laboratories), the community itself has lacked the organization and management that might provide this guidance for funding priorities and policies and be applicable to the entire spectrum of marine research.

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The National Association of Marine Laboratories: A Connected Web for Studying Long-Term Changes in U. S. Coastal and Marine Waters

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Abstract. Long-term time-series measurements provide data that test specific hypotheses or suggest new avenues of study. Such studies are widely acknowledged as important for differentiating the influence of human activities from natural background variability. Several long-term research or monitoring programs are active in coastal and marine environments around the world and serve as models for development of new studies. The spatial array of U.S. coastal laboratories is suitable for resolving latitudinal trends and for many types of comparative studies. However, establishing a network of coastal laboratories focused on long-term monitoring and research problems presents special challenges in setting research priorities at appropriate scales, in data management, and in coordination of the scientific effort. The National Association of Marine Laboratories (NAML) is uniquely positioned to promote long-term studies among networks of its member institutions. The NAML can play an effective role in publicizing the importance of long-term studies, in providing access to expertise in this type of research, and in promoting its continuance for periods longer than the length of individual scientific careers.

Preface

"The goals of NAML are to:

—advance the wise use and conservation of marine and coastal habitats and resources;

—encourage and support the recognition of the unique role of coastal laboratories in competent environmental research, academic and outreach education, and public service;

—promote efficient information exchange, constructive cooperation, and productive coordinated effort among member institutions;

—provide a contact and forum for efficient exchange of information and utilization of expertise between member institutions and governmental agencies."

National Association of Marine Laboratories promotional brochure, *circa* 1990

Introduction

Long-term measurements of ecological and physical phenomena are, arguably, the most valuable data we collect for understanding how the world works and how it changes over time, whether naturally or under the influence of human activity. Examples of such data are few but very well-known, and they have been the foundation of research in which the dynamics of processes contributing to environmental changes are measured. Studies conducted under the rubric of "global change" can probably all be related in some way to the continuing, nearly 40-year-long collection of atmospheric CO_2 data initiated by Keeling in 1958 at the Mauna Loa Observatory in Hawaii (Keeling *et al.*, 1976). The broadscale fishery and climatic events modeled by Sharp and McLain (1993) would have been difficult to describe without the long-term measurements of the "southern oscillation" in the equatorial Pacific Ocean by Troup (1965). Such climate-driven events in the ocean or on land simply cannot be understood unless they are put into the long-term context (Peterson *et al.*, 1995; Plaut *et al.*, 1995). Thus, natural processes or phenomena that change with time—whether slowly or rapidly, periodically or irregularly—cannot be documented reliably from a single field expedition; in such cases, repeated oceanographic measurements are imperative. Time-series studies are also ideally suited for the documentation of complex natural phenomena that are under the combined influence of physical, chemical, and biological factors [*e.g.*, the recognition of acid rain at Hubbard Brook by Likens *et al.* (1977)]. The valuable legacy of many other long-term biological studies is aptly noted in several recent publications (*e.g.*, Champ *et al.*, 1987; Spellerberg, 1991; Kramer, 1994; Leigh and Johnston, 1994). Data derived from the few other extant long-term oceanic time-series provides ample incentive and scientific justification for establishing additional study sites (Wiebe *et al.*, 1987).

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This paper was originally presented at a workshop titled *Roles of Coastal Laboratories in the Implementation of the Nation's Emerging Priorities for Research in the Coastal Zone.* The workshop, organized by the National Association of Marine Laboratories, was held in Sarasota, Florida, from 24 to 27 October 1995.

Given the immense value of carefully constructed time-series measurements, how can the diverse group of American coastal laboratories—geographically separated, only partially connected through meetings, telephone, facsimile, and electronic mail, physically different and with vastly different monitoring and research capabilities—be organized for long-term monitoring in the coastal and marine environment? What role can the National Association of Marine Laboratories (NAML) play in facilitating this important task?

This paper identifies several aspects of long-term studies that should be considered before a coordinated monitoring and research effort can be promoted or facilitated by the NAML organization and undertaken by groups of coastal laboratories. We indicate some problems that might be addressed by a coordinated effort organized with NAML support and recommend some potentially viable approaches. Several current model programs with similar goals in place are examined as case studies. Lastly, we propose several types of activities that the NAML organization can do to initiate the coordination process.

Some Considerations

Both scientific and logistical considerations influence the establishment of any long-term time-series measurement program. Foremost among these are site selection, choice of variables to be measured, sampling design, and sampling frequency. Equally important is the assessment of the desired accuracy and precision, which strongly influences the choice of the analytical method to be used for a given candidate variable. Other project design considerations include the availability of suitable reference materials, the hierarchy of sampling replication, and knowledge of the magnitude of mesoscale horizontal variability for data collected at a fixed geographical location, *i.e.*, whether values measured for variables are similar or greatly different at nearby stations. In addition, common methods must be employed to ensure that data collected will be strictly comparable.

Long-term time-series research programs present special problems for scientists in general (Strayer *et al.*, 1986) and for marine scientists in particular (Tabata, 1965; Wolfe *et al.*, 1987). Besides sufficient funding to maintain these costly programs and their attendant personnel management and logistical concerns, there have been misconceptions and thus negative attitudes among some scientists and funding agencies about the value of "environmental monitoring" (Karl and Winn, 1991). Experience has shown that antecedent data collection is necessary and worth the investment, as it is impossible to presage the types of interesting hypotheses that emerge over time from monitoring efforts. It is also very important that every program incorporating time-series observations be fully integrated into experimental and theoretical frameworks and involve scientists of the highest caliber. Finally, many ecological processes are complex and involve time lags, threshold effects, and both positive and negative feedbacks. Consequently, the effects of these processes on populations and habitat variability may not easily be revealed through simple statistical analyses of observations. Effective data assimilation and modeling are clearly vital to the long-term success of these monitoring programs. Thus whether conceived of as valuable for their future broadscale applications, or as a means of answering more local questions, long-term studies have had, and will continue to have, ever greater implications for science and society.

What Types of Studies Are Appropriate for Collaboration Among Coastal Laboratories?

Many topics are suitable for hypothesis-driven questions: *e.g.*, the effects of changing land use patterns and their point source and diffuse inputs on water, sediment, and habitat quality; the effects of large-scale removal of higher trophic levels (fishes) on ecosystem dynamics; sea level rise impacts on coastal habitats; and our abilities to better define biogeographic boundaries in the context of physical and climatic processes on broad spatial scales. Individual coastal laboratories might also be expected to have very different degrees of "pollution," and this could be the variable of interest.

In terms of long-term monitoring, many approaches are possible, including such relatively easy things to measure as surface water temperature and salinity or even *E. coli* counts. What we don't have are data on processes that can be linked and compared across habitat types represented at each laboratory's location. A simple measurement of surface water BOD (biological oxygen demand) integrates several dynamic processes with implications for water quality and coastal ocean health. The same can be said for something like Secchi disc depth measurements taken wherever the water is, for instance, ten meters deep. Such observations could be linked with data from other governmental agencies that monitor stream and river flows with broad implications for measuring erosional losses on a national scale.

In terms of long-term research, there are an equally large number of possibilities. The best approach would be to assemble teams of laboratories with unique and comparable capabilities to examine local or regional questions. For instance, those with strong plankton sampling capacities could look at representative taxa and measure their lipid content and body burden of pesticides or their rates of pesticide uptake. This could lead to an examination of pesticide degradation rates, for example. Those labs with strong capabilities in chemistry might create a matrix of spatial and temporal observations on nutrient concentrations with a look towards the capacity of the coastal region for absorbing those types of human impacts. Even labs with only modest capabilities in these areas could at least contribute samples to the mainstream labs for whatever question was under study.

The following example illustrates the immense power of long-term collaborative networking. Determining the rates at which bacteria use dissolved organic matter in waters of differing salinity does not require a long-term study. However, determining the relative amount of bacterial biomass in coastal waters as a function of periodic fresh water discharge requires a much longer period of data collection. If the question is whether the bacterial response is similar at all latitudes, then a spatial array of observations is necessary. This is because the magnitude or scale of the riverine discharge may be different at each coastal site, and the pattern of discharge may also differ due to timing of storm events. Higher latitude sites may have a prolonged period of runoff in the springtime, whereas seasonality in runoff volume may be subdued or absent at tropical sites. Obviously a coordinated effort among latitudinally dispersed facilities such as coastal laboratories would be required to approach this type of study, whether to look at bacterial contamination of coastal waters in a public health sense, or to answer related questions about rates of nutrient regeneration or sediment input as they change through time.

The example above also points to one of the unique aspects of the NAML membership—the organization includes an inherently beneficial spatial array of coastal laboratories having latitudinal and other gradients (*e.g.*, watershed size, population density, etc.) along our coastlines. No ship or cruise plan can possibly duplicate what could be done synoptically within the NAML organization. The potential for new knowledge derived from cross-site comparison of common data sets is enormous. This is but one of many advantages (Table I). Unfortunately, not all laboratories are equally capable of participating in a coordinated long-term study (Table II). The mere size of coastal laboratories (and their highly correlated annual budgets) partially reflects the scale of operations that each can support, but the critical aspect for any laboratory is the long-term component of the activity. Even adequate funding cannot assure continuity of trained personnel to perform the measurements or of the required intellectual leadership.

Data Management—A Critical Requirement

Many institutions with long-term studies underway are successful because they have site continuity and integrity, staff stability with relatively low turnover, a commitment from their higher administration to maintain the long-term effort, and science-driven questions that the long-term data are designed to address. These attributes have been extolled previously by Wolfe *et al.* (1987), but the issue of data management was only partially addressed by these authors.

Table I

Aspects of U.S. coastal laboratories that are favorable for creation of a coordinated long-term program of monitoring and research

A. Location
   - Present on all coasts in all coastal states
   - Cover a broad range of latitude and longitude
   - Comprise diverse habitat types: open coast, sheltered bays, estuaries, coral reefs, clear and turbid waters, hard and soft-bottoms, deep and shallow waters, etc.
   - Accessible by land and air as well as by sea
   - Under varying degrees of anthropogenic stress: tourism, industry, aeolian deposition, etc.
   - Can include large lake systems

B. Physical Plant
   - Docks & piers provide easy access to shallow coastal waters
   - Boats provide access to nearshore habitats
   - Analytical equipment in place for a variety of physical and/or chemical and biological measurements
   - Archival space for samples may be available
   - Communications facilitites: phone, fax, e-mail, newsletters
   - Meteorological capabilities for weather observations

C. Personnel and Technical Staff
   - Highly skilled or specialized people who know the local fauna, flora, and conditions
   - Taxonomic expertise

D. Resources
   - Private, local, state, federal funding available
   - Volunteer helpers from local area
   - Partnerships with various interests

Table II

Aspects of coastal laboratories that impair their abilities to perform long-term projects

- A. Location
  - Study sites may be difficult to access on a frequent or regular basis
  - Seasonal weather patterns may preclude observations
- B. Physical Plant
  - Maintenance of critical facilities (especially meteorological stations)
  - Loss of facilities to storms or other disturbances
  - No capability for sample storage
  - No data management capabilities
- C. Personnel and Technical Staff
  - Lack of personnel or lack of skilled personnel
  - Normal turnover and training of replacements
  - Competition with other job obligations
- D. Resources
  - Lack of funds to purchase necessary supplies
  - Changing priorities, reductions in force
  - Partnerships dissolve
  - Failure of leadership

Data management is an integral part of long-term studies, a component that becomes more important and often more demanding of resources with time. As a study progresses and the data and its attendant metadata (documentation describing the data) accumulate, quality assurance routines and data management requirements grow as well. Hardware, software, and storage devices reconfigure and require updating every two or three years. Unfortunately, these costs preclude effective data management at many smaller coastal laboratories. However, the lack of sufficient funds to perform data management will not be a tenable argument in the future because (1) external funding will be tied to demonstrated, effective data management procedures; (2) data management costs can be included in proposal budgets; (3) data management, if creative, can be done inexpensively; and (4) the long-term benefits outweigh the costs.

In a coordinated long-term study or monitoring project involving several institutions, some laboratories will be able to perform effective data management, while others will not. A possible and obvious solution is to centralize data management at a few institutions (or even a single site) having the necessary infrastructure and communication capabilities to do the job while providing associated decentralized access throughout the network. Without attention to data management on-site or at such a centralized facility, a coordinated long-term research or monitoring program for coastal laboratories is doomed to failure.

One model for the management of standardized data collected at several different sites is the Centralized Data Management Office (CDMO) of the National Estuarine Research Reserve System (NERRS). Research at the 22 individual reserves in this program is managed locally by personnel at marine laboratories from throughout the nation and Puerto Rico. As part of a system-wide, long-term environmental monitoring program (the NERRS National Monitoring Program), each reserve site collects water quality data (dissolved oxygen, temperature, salinity, nutrients, etc.) with mechanized data loggers deployed *in situ* at locations representative of problem areas. For example, the water collectors may be positioned in small tributaries that drain agricultural basins or in areas whose waters have a high residence time. Data are downloaded to computers at each site where individual reserves are responsible for quality assurance, quality control, and documentation. Data and metadata are then transmitted electronically to the CDMO computer hub for storage, graphic representation in standard formats and scales, and other standardized manipulations. Each data-generating site has access to its own data or data from any other site, *i.e.*, it is a shared database. The centralized network office of the Long-Term Ecological Research (LTER) program works basically the same way, except that data are not collected with the same instruments at the same frequency, and no two sites may be collecting all of the same types of data (Franklin *et al.*, 1990). The U.S. Joint Global Ocean Flux Study (U.S. JGOFS), on the other hand, has a distributed data management scheme (Brown, 1992).

Another issue—data ownership (Wakefield, 1995)—is handled in the LTER network by giving data generators exclusive rights to publish their own data for an appropriate, agreed upon period, at which time it becomes available to the public. The exclusive period is usually one or two years but can be quite variable in length, depending upon site and investigator needs. It is one year for both U.S. JGOFS (core data sets only) and the NERRS National Monitoring Program. The latter has a formal data ownership and dissemination policy that allows a one-year exclusive period for principal investigators (D. Porter, pers. comm.). This mechanism ensures that file cabinets and personal computers do not fill up with data that will eventually be lost, but enforcement of the policy and promulgation of timely publication requires a dedicated effort by principal investigators and data managers at each LTER site as well as by those at the LTER network office. Having a standardized data collection protocol would reduce the need for extensive on-site data management, as a centralized database compiled using common methods would be accessible to all.

Which coastal laboratory would be willing and able to perform this task for a NAML-organized network? Because the NAML membership is regionalized, one or two laboratories in each region could perform data management activities for any group of laboratories in the region having similar data collection needs. Coastal laboratories could also organize across regions for specific research questions. This presumes, of course, that the regional group of laboratories can agree to attack a question requiring standardized collection of data over a long time period. This aspect itself is probably a more difficult issue to resolve than mere data management, but as Nixon (1996) points out, coastal and marine laboratories may have to approach coastal zone management issues on a regional basis if they are to survive.

Establishing Appropriate Long-Term Research Priorities

In thinking about the sorts of coastline-scale long-term questions that should be approached through monitoring studies, it is appropriate to reflect on why there are so many different coastal and marine laboratories in the first place. The roles of these institutions are as varied as their sizes and locations, but a common thread is their local, regional, or national community service as a way to advance education, research, and public outreach (see Preface; *Am. Zool.,* 1988). Every laboratory touches at least some aspect of these services, and their locations historically have also provided each a "sense of place," a concept developed more thoroughly by Tenore (1996). Therefore, we should ask ourselves how the long-term collection of data can fit within the broader mission of coastal and marine laboratories to serve the public. These services are provided in both direct and indirect ways.

Direct service involves such things as K-12 teacher educational programs about marine ecology, advice on disease prevention to managers of shrimp ponds or oyster farms, coordination with county council members about siting problems for local industries or municipal sewage outfalls, or involvement in management decisions concerning health-related closure of public beaches or shellfish beds.

Indirect service comes from basic research activities that have applied outcomes. For instance, the results of long-term studies on nutrient fluxes within a watershed can be applied to management of runoff in local soil management districts. Studies on larval recruitment can be applied to decisions about when to open or close fishing or shellfishing seasons (*e.g.,* DeLancey *et al.,* 1994). Thus the real challenge is to establish a set of hypotheses that can be tested only at the regional or national level that will have implications for the public good. The public is not always willing to wait for answers to long-term questions, but they (and funding agencies) will respond positively as long as coastal laboratories—through NAML—put effort into effective public communication about the intrinsic value of their research. An editorial in *Science* bolsters this view (Woolley, 1995), and a recent workshop designed to establish program objectives for the newly created NOAA Coastal Services Center in Charleston, South Carolina, clearly identified issues for study which the public will embrace.

Time and Space Domains: The Matter of Scale

The coastal ocean exhibits natural time and space variability on many scales, ranging from millimeters to kilometers, seconds to decades, or more. Establishing only a single time-series sampling site eliminates from further consideration the important spatial component of habitat variability. But an interconnected web of otherwise independent geographical sites, as could be organized by the NAML, could provide critical data on both the space and time components of coastal or oceanic variability.

Therefore, once again the matter of observational scale—spatial and temporal—becomes relevant (Williams, 1984; Livingston, 1987; Levin, 1992; Giller *et al.,* 1994). Melding spatial and temporal variability in an ecological or physical research project is a fairly routine matter (Mann and Lazier, 1991). The more difficult task is dissecting and differentiating among the effects of space and time upon our results. Studies that examine scale in either domain are fairly recent arrivals in the marine science literature. Large-scale patterns were frequently the result of wholesale extrapolation based on too few sampling stations. Classic examples are the early work on zooplanktonic biomass distribution patterns in the Pacific Ocean (*e.g.,* Vinogradov, 1970) and early maps of primary production in the sea by Koblentz-Mishke *et al.* (1970). All this guesswork about patterns has changed now: synoptic observations at increasingly larger spatial scales and over increasingly longer time periods are almost viewed as routine with the help of satellites, moored instrument platforms, acoustic tomography, and dataloggers of all types. The computer "revolution" that has enabled us to assimilate, portray, interpret, and understand massive amounts of data has indeed revolutionized our capabilities for examining scale and pattern (Platt and Sathyendranath, 1988).

Another important issue in long-term, time-series studies is consideration of how long is "long-term," and how long is data collection necessary and sufficient to test a particular hypothesis. A decade-long data set can be used to assess interannual variability but will have little success in resolving the effects of quasi-periodic phenomena like the El Niño—Southern Oscillation that has temporal scales of 4–7 years. As Green (1984) so aptly states, our science has hopefully matured enough to accept that some critical problems will simply require long-term research investments.

What have we learned about scale? Both the size of the study area and the duration of an experiment can affect the data and thus our view of the phenomenon under study. Moreover, this relationship holds whether the investigation involves an individual organism, a population in a patch, an area large enough to link patches via dispersal mechanisms, an entire biome, or a biogeographic range of diverse habitat types. Disturbance events affecting each scale are likely to be of longer duration as the study area increases in size; *e.g.,* tornadoes as opposed to hurricanes. Whether emergent properties related to scale at the community level are sufficiently well-known that we can generalize from them is still open to debate, because each interacting population within a community may be operating at a distinctly different scale whose dimensions have been defined evolutionarily. Therefore scale effects depend on the nature of the question under investigation. As we continue to examine processes at various scales in the sea—currently being done in the National Science Foundation's U.S. Joint Global Ocean Flux Study (Fig. 1)—we can expect new properties to surface and new principles to emerge. A comprehensive understanding of natural habitat variability and the ability to resolve natural from human-induced change is likely to require several decades of observations. In addition, the temporal scale of planned observations must match the spectrum of available resources—both fiscal and intellectual. The question of scale remains a worthwhile, testable target for every discipline in the aquatic sciences that can easily be attacked with long-term studies at coastal and marine laboratories.

Other Programs in Place: A Unique Role for the NAML

There are several long-term research or monitoring programs in place throughout the world: *e.g.,* France's National Research Project on Coastal Zones (PNOC, 1995), and many in the United Kingdom (Spellerberg, 1991; Gamble, 1994). Some have evolved from small-scale efforts whereas others have been conceived as long-term investments from their inception. Long-term data have been collected in European countries for much longer than elsewhere, but the more recent efforts have, with a few notable exceptions, been better organized conceptually and managerially [*e.g.,* LTER (1993), and LMER (Hayden *et al.,* 1991); EPA (1994a, b); NERRS (1992; Crosby and Beck, 1995)]. The widespread media reporting of global warming, ozone thinning, sea level rising, coral reef bleaching, seals dying, and climate-related coastal erosion is helping to position us, at just the right time, to contribute to managing our long-term investment in preserving oceanic, neritic, and coastal habitat.

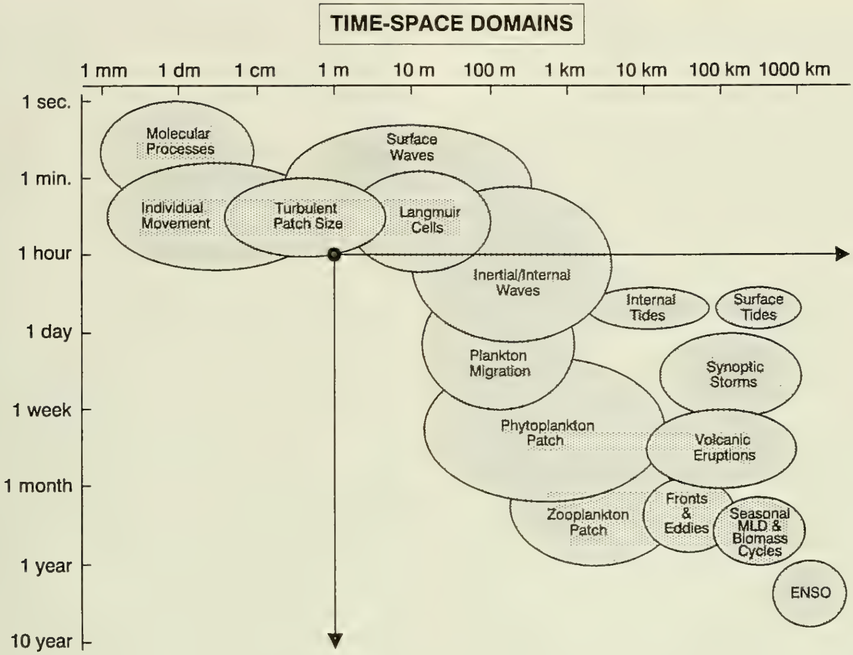
We must not compete with other efforts in progress or underway. Similarities among the goals and working group recommendations for creating ILTER (International Long-term Ecological Research) programs, for enhancing communications among members of the Ecological Society of America (Colwell, 1995), and other ideas about linking non-coastal biological field stations with coastal laboratories in a national network (Lohr *et al.,* 1995) should form a strong basis for, and justification of, any coordinated long-term program that results from efforts of laboratories in the NAML membership.

A Contrarian Viewpoint

Although the Internet is available globally now, it does not provide the only appropriate or useful means of forming coordinated links among marine laboratories for research and monitoring purposes. Therefore, as software and hardware technologies improve, other mechanisms for linking laboratories should also be sought (*e.g.,* satellite-based, real-time video communications). This challenge is not trivial, because so many technological decisions will need to be made (beyond the difficult scientific questions) about the standardization and structure of data collection, physical storage devices, information dissemination modes, user fee support, and other data management issues. What would be the cost of getting every laboratory properly calibrated to measure salinity and temperature routinely and to contribute time-series measurements to a centralized (or distributed) database? Will the effort to coordinate such a diverse group of laboratories be worthwhile? Can any laboratory afford to divert and refocus already scarce resources towards participation in a networked long-term monitoring or research program? How soon might new monies become available for such programs as this?

A few examples may shed light on the immensity of such an exercise. The LTER budget (with many fewer sites than NAML has laboratories) is about $10 M per year. The LMER budget is about half that, and the NERRS budget is less, about $4 M. Therefore, a NAML-scale program might cost about $10 M at a minimum. How many funding sources would be needed to accumulate such a sum? These are easily asked questions, but absent a coordinated plan, answers are not defensible.

At least one formal group, the Mid-Atlantic Regional Marine Research Program, has examined some of these technical problems with a view toward creating a coordinated regional data management plan (Antech Systems, 1995). The Atlantic States Marine Fisheries Commission has published results of a workshop held specifically for coordinating use of extant long-term bottom trawl survey data (some since the early 1970s) for fisheries management (Berger, 1994). It is instructive, therefore, to ask what the NAML can (or should) do beyond these more local or regional efforts. Should we pursue anything on a national scale? Have we maximized the amount of science being done per unit effort, or is there room for redirection?


TIME-SPACE DOMAINS
1 mm
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1 min.
1 hour
1 day
1 week
1 month
1 year
10 year
Molecular Processes
Surface Waves
Individual Movement
Turbulent Patch Size
Langmuir Cells
Inertial/Internal Waves
Internal Tides
Surface Tides
Plankton Migration
Synoptic Storms
Phytoplankton Patch
Volcanic Eruptions
Zooplankton Patch
Fronts & Eddies
Seasonal MLD & Biomass Cycles
ENSO

Figure 1. Temporal and spatial domains of various physical and biological processes in the sea (adapted from Dickey, 1990).

Role of the NAML in Organizing a Network of Laboratories

Perhaps the first action item for NAML is to initiate and organize a survey of the long-term, time series parameters currently being measured at coastal laboratories. Information should include why the measurements are being made, their methodologies, sampling locations, measurement frequency, study duration, and the types of quality control measures in place.

The NAML organization should also establish a registry of experts on various aspects of long-term studies to provide advice to laboratories and other groups preparing to engage in time-series types of measurements. Such advice might include specific information on data management and data dissemination as well as on methods for ensuring high quality measurements. The thrust of this registry would be not only to provide advice, but also to enable an assurance that environmental policy decisions and management actions resulting from monitoring activities can be based on the highest quality data and trend information.

The length of many important time-series measurements will exceed the length of the scientific careers of individual scientists who initiate them. The NAML, through networking and consensus, should advocate continuance of these efforts whenever these motivated scientists retire. This will, of course, require substantial leadership and support for long-term efforts, and the NAML can promote the rationale by showcasing some long-term data sets that have had a significant impact on science and society. Previously cited examples of the CALCOFI (California Cooperative Fisheries Investigations) data sets linking ocean warming and a major decline in zooplankton abundance (Roemmich and McGowan, 1995a,b), the long-term plankton records for the North Atlantic showing climatic trends on biota (Gamble, 1994), and familiar freshwater lake cleanup success stories like Spirit Lake (Larson, 1993) and Lake Washington (Edmondson and Lehman, 1981) all provide fodder for that rationale.

The serious effects of overfishing, whether on George's Bank cod or oysters in Chesapeake Bay, also provide an opportunity for the NAML to promote the utility of long-term studies by showing how they provide the data necessary to discover these disturbing trends. The popular press has a willing and receptive audience for such accounts (*e.g.*, Parfit, 1995; Safina, 1995), and we must seize the opportunity to promote the NAML's role in producing these long-term studies of discovery and in keeping our citizens informed through research, education, and outreach.

There is a clear need for long-term, high resolution time series measurements of key environmental and biological variables at strategic locations in representative systems. Given their diverse capabilities, the NAML's coastal laboratories have great potential for long-term and large-scale monitoring and research. However, until a coordinated network is established, much of this potential will never be realized. This is the organizational challenge that faces the National Association of Marine Laboratories.

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Integrating Research and Education with Public Outreach at Coastal Laboratories

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Abstract. Coastal marine and Great Lakes laboratories increasingly are asked to provide both advisory and educational outreach to the general (and often specific) public. To facilitate this interchange, basic and applied research must be more integrated with advisory services, with care taken to present facts and concepts, not opinions or advocacy. Moreover, outreach efforts should be proactive, not reactive. With the rapid expansion of telecommunications, such as electronic mail and teleconferencing, outreach can optimize the links between education and research.

Public outreach also gives graduate students an opportunity to utilize practical applications and interpretations of marine science, thus providing valuable experience that can help in obtaining future jobs. More problematic is how outreach activities can be evaluated in an annual or promotion review of a faculty member; particular care must be taken not confuse outreach with scholarship, or sacrifice intellectual rigor, in such evaluations.

Introduction

Marine laboratories and departments in recent years have been asked to provide the public sector with an ever increasing amount of advice and knowledge about the marine environment. In part this reflects an increased public awareness of the environment and society's interdependence upon it. At the same time, marine laboratories have more information to share, because the opportunities for coastal research have expanded in recent years.

The end of the Cold War and the subsequent diminished Soviet military threat meant a major shift in defense-related research (see Wunsch, 1993, for an insightful discussion). No longer do our immediate security threats come from countries with formidable naval fleets; rather, future conflicts may well involve coastal operations—mine warfare, landing craft operations, and subsequent impacts on harbors and coastal waters. As a result, much of the national defense research has shifted from deep-water acoustics, water circulation, and bottom morphology to coastal problems such as shelf dynamics. At the same time, coastal research programs in other agencies have increased substantially: *e.g.*, Coastal Ocean Program (CoOP), Land-Margin Ecosystem Research (LMER), Shelf Edge Exchange Processes (SEEP), etc. Thus, coincident with an increased public concern for the coastal ocean, researchers and educators are shifting their attention coastward.

This paper addresses the role of coastal marine laboratories in public outreach activities. What types of activities are involved, how can they be optimized, and how should we evaluate faculty activities in outreach programs? I prescribe no solutions, but I try to define questions that each coastal laboratory should consider.

Public Outreach and New Roles for Coastal Laboratories

By its very name, outreach defines the proffering of experience or advice to the general public from an expert, in this case the research marine scientist or extension agent. Implicit in the word outreach is that it most often is proactive, but my colleague Bill DuPaul has found that only about 60 percent of the advisory work at the Virginia Institute of Marine Science (VIMS) is planned; the rest is reactive to unforeseen problems and opportunities. Out-

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reach also should be scientific in basis and unbiased in approach. Moreover, by its very nature and clientele, public outreach generally addresses short-term, local problems rather than longer term, global problems. Thus, beach erosion and aquaculture are more likely outreach concerns than is global climate change, although global problems may be intimately involved in local problems, such as the role of global climate change in the local rise of sea level.

As I define the term, outreach encompasses two distinct types of activities. Although both involve the transfer of scientific or technological knowledge, *advisory services* can lead to a practical or economic outcome, whereas *educational outreach* most often involves information transfer for its own sake. Advisory services should transfer knowledge in a form useful to people, encourage appropriate adoption of this knowledge, and stimulate others to carry out research to solve coastal problems (NOAA, 1978). Examples of advisory services are numerous—helping in fishing trawl design, advice on aquaculture, conservation assistance, local or state planning, etc., but most are aimed at encouraging a change in the client's method of operation. A proposed harbor development, for example, might be reconsidered if it is concluded that dredging will affect local fisheries; the mesh size of trawl nets may be increased if this increases the stock of fecund females, etc.

Educational outreach can include teaching students in kindergarten through high school or teaching their teachers, many of whom either have had no formal science training themselves or whose scientific education is dated. Special audiences also can use educational outreach, *e.g.*, clubs, Elderhostel. Finally, educational outreach can include answering *ad hoc* questions by the curious public—"I found this strange shell today . . ."

In the early years of U.S. higher education, most college and university faculty members were primarily concerned with teaching, not research. But the passage of the first and second Morrill Land-Grant Acts in 1862 and 1890, which led to the establishment and funding of Land-Grant colleges, raised the expectation that faculty at these institutions would also participate in extension services, that is, provide service to their constituents. By the turn of the century, basic research at many universities was being emphasized, often at the expense of teaching, and public service as a normal faculty activity was de-emphasized (Boyer, 1990). It is only recently that such concepts as "service", "outreach" and "extension and advisory services" have been championed once again, perhaps in part because of the public's increased concern about how funds are spent.

To most of us this renewed emphasis on outreach seems to have occurred almost overnight. For instance, could the following words have been accepted and incorporated into college and university strategic plans even 20 years ago?

> Goal: To make public service a more visible part of the curriculum.
> Goal: To increase student participation in public service activities.
> Goal: To increase the visibility and influence of public service . . . and extend the reach of the College's public service activities.
>
> (College of William and Mary, 1995)

or:

> Advisory service is recognized as a fundamental feature . . . that is dependent upon, integrated with, and supported by the contribution that institutional research and scholarship makes to its content, quality and value. Advisory services should strive to be proactive and anticipatory . . . Advisory service is also of considerable value to the graduate program, where it offers unique educational opportunities.
>
> (VIMS, 1995)

In contrast to the last century, when many practical questions revolved around farming, extension services provided by colleges and universities now encompass a far greater range of needs and interests. As the number of coastal inhabitants increases, for instance, it is not surprising that there is a corresponding increased interest in water and how mankind lives with it.

Advisory Services—Linking Basic and Applied Research

A rather simple truism often overlooked by researchers and managers is that most good basic research has practical application, and, conversely, effective applied research necessitates a solid grounding in basic research; that is, applied research can lead to new understandings of basic relationships. A clear example is coastal and estuarine research in which there is a close correlation between scientific problems and management concerns, as seen in a list of scientific research priorities in future coastal ecosystem research (National Research Council, 1994):

- Develop indicators of biological status and processes that reflect ecosystem health and integrity;
- Define better the relationship between physical phenomena and ecosystem structure and function, and utilize *in situ* observation systems to provide better insights into ecosystem behavior on various time and space scales;
- Understand better the interactions and linkages between atmosphere, watershed and coastal ecosystems,

and the effects of modifications to land-use and water flow upon transformations to the watershed and coastal habitat;

- Use research, modeling, and monitoring to support effective habitat restoration or rehabilitation.

In each task one recognizes immediate practical problems that correlate with scientific problems. Said another way, can one imagine effective management of an estuary or coastal area without a basic understanding of these scientific phenomena?

As with land-grant universities, much of the ability of marine laboratories to pursue applied research depends on federal funding, often from the National Sea Grant College Program. However, the Department of Commerce (DOC), the National Oceanic and Atmospheric Administration (NOAA) and its agency, Sea Grant, all face increasingly uncertain futures. In recent months DOC and NOAA have been threatened with elimination by various House and Senate committees. Sea Grant's budget always seems to be at the edge of drastic reduction, and the program's survival (with increased funding) is ample evidence of its success. But while Sea Grant Marine Advisory Service programs have continued to grow, most of that growth has come from matching funds (27% increase between 1984–89) rather than federal Sea Grant funding (2% for the same period) (UNC, 1992). Moreover, most researchers often need significantly more money than the Sea Grant budget can provide, and Sea Grant proposal review often is politicized. These problems can drive good researchers to other avenues of funding, one result being that advisory service may become a casualty.

What is the client base for advisory services?

In coastal work, the clientele includes coastal planners (including port development and marine transportation), the food industry (fishermen, aquaculture, and processing), non-living resources (such as recreation and mining), conservation (including solid waste disposal and coastal erosion), as well as persons concerned with such diverse topics as exotic and endangered species (NOAA, 1978; National Sea Grant Program, 1991). Clearly the types of outreach and advisory activities depend greatly on the institution involved. A large private lab, for instance, may have no local or state constituency upon which it can rely for some of its financial support. In contrast, many state-supported laboratories may include advisory activities in their formal mandates.

The scientist's audience often is defined by the source of his or her funding. Should a scientist relying solely on NSF and ONR grants necessarily be expected to respond as quickly to a local outreach request as a scientist funded by the state? In the latter case, the scientist is responding to his "funding agency"; in the former case, the scientist's constituency is different, although an NSF-funded scientist is expected to help in proposal reviews and perhaps participate in NSF review panels. A somewhat more subtle problem may arise at a university where a major benefactor may expect (and receive) a faster and more thorough response than someone who has no such university connection.

Many state-funded coastal laboratories have advisory services that are closely analogous to agricultural extension services at Land-Grant universities. Those supplying information may be faculty members or extension agents, often with long-term relationships with specific client groups. Advice can be given to for-profit operations, such as fishermen and aquaculture growers, or to policy makers and implementers, such as planning boards, state or local agencies, and legislative bodies.

Many forces that influence policy decisions are not based on technical facts as scientists might construe them. While it is the responsibility of the scientist to offer the best possible advice, the scientist also must realize that the politician must pay heed to his or her constituency. Said another way, the ultimate decision maker needs to listen to many, but may follow advice based on non-scientific factors, such as economics and politics. A fisheries scientist might conclude that a certain species is in danger of being overfished. He recommends a moratorium, but the moratorium may mean that fishermen will be financially impacted. Hence the moratorium is not implemented.

To be most effective, advisors must be viewed as non-advocates—they impact knowledge, not opinions (NOAA, 1978). If perceived as representing an unbiased viewpoint, one's perspective or advice may be followed more readily than if the decision-maker views the advice as coming from an advocate. In other words, advisory service should lead to informed decision making.

Optimizing advisory service delivery

So far, I have discussed mostly advisory services in which the client requests help, either directly or indirectly. But the conveyance of information also can be initiated by the scientist. This goes beyond educational outreach, which is discussed in the following sections, for it deals with how the public receives, understands and relates to research programs at marine laboratories. A sound outreach effort necessitates a logical structure and flow diagram, one example of which is seen in Figure 1. But the linkage between the provider and the client often requires a facilitator—individual scientists cannot be expected to field any and all questions, at least not effectively.

At many laboratories, calls of interest or questions are answered initially by the switchboard operator, and therefore the call may not be transferred to the most appropriate

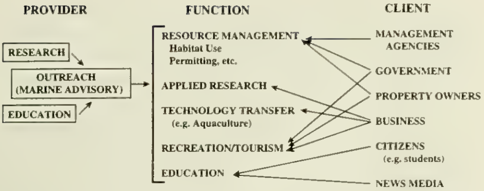

Figure 1. The outreach and marine advisory office at a marine laboratory can provide the vital link between the outreach and educational activities and the client who requests these services. The actual provider, of course, depends on the function required as well as the particular client.

staff member. A more logical (if expensive) way is to have a public information office that is sufficiently linked to the research activities of the laboratory so that it can forward any question to the proper person or office immediately; callers do not like to be referred to a string of offices, particularly if none has answered the request.

Once a request is received and the information provider is assigned, the method and time of response should be identified, so that conflicting messages are avoided. Can the question be handled simply over the phone (*e.g.*, "That dead mass on your front lawn, Madame, is probably a horseshoe crab torn up during the last storm.") or does it require a site visit or time in the library or laboratory? Will it necessitate a simple oral report, a written report, or an appearance at one or more hearings? As Healy and Hennessey (1994) point out, all too often scientists play only episodic roles in resource management science, whereas a more effective method is to have science involved throughout the management process. Unfortunately, the most effective advice may require a great deal more time (*i.e.*, becoming proactive rather than reactive) than many busy scientists are willing to spend.

It almost goes without saying, but the advice should be given in terms that are easily comprehended by the layman. Even the simplest scientific concepts, however, can prove daunting to the lay person, particularly if the science answer is laced with jargon. Providing incredible insights and sage advice do little good if the user can't understand what is being said.

Inherent in advising and extension service, of course, is the follow-up. What was the outcome of your advice? Was the client's ultimate action successful? Is further work anticipated, and (less altruistically) might this work lead to research opportunities for the scientist or the laboratory?

Working with the media

The fastest way to reach the public is through the news media. Many marine laboratories have a public information officer through whom media requests for information or interviews can filter. But whether buffered by an information office or not, most scientists have been interviewed by a reporter whose published article misconveyed the scientist's research or message. Where the news is not "fast-breaking," the sympathetic reporter might allow the scientist to read a draft of the article for technical comments. But where an important story cannot wait, the scientist must present the clearest account to the reporter, knowing that the reporter may have little understanding of the actual underlying science.

Institutions can have media open houses or workshops, where media representatives learn about the activities of the marine laboratory in a more informal manner. The Marine Biological Laboratory in Woods Hole runs a Science Writing Fellowship Program each summer. Alternatively, scientists can learn how to communicate more effectively with the news media, and a number of scientific organizations have begun to sponsor media and communications programs. The 1996 Ocean Sciences Meeting in San Diego, for instance, offered a short course in communicating with the media, which included practice interviews in which the scientist was questioned by a mock reporter. One piece of advice given in the article announcing this short course (EOS, 1996, v. 77, p. 20) is that the scientist should not feel compelled to supply an immediate answer to all questions; taking an hour or so to prepare an answer is usually okay.

One can, of course, always write one's own article. Semipopular magazines provide one avenue, the best example being Woods Hole Oceanographic Institution's *Oceanus,* which, for many years, has provided the educated laypublic with clear and concise ideas of the marine environment and how scientists study it. Recently, however, *Oceanus's* focus has been redirected to concentrate on the scientific research conducted at WHOI.

With the recent explosion in electronic communications, the scientist now can be as close to the public and the media as his or her PC keyboard. One can hold teleconferences at which questions and answers can be immediately communicated. Electronic mail allows one to answer questions and requests in the leisure of one's office, and features like the Internet and World Wide Web provide public access to general information about the institution's general programs as well as recent scientific projects and results. This increased communication along the "Information Highway" can greatly enhance public access as well as facilitate rapid feedback. As both the need to publicize one's research and the access to rapid electronic communications increase, however, one could be tempted to confuse the media with peer-reviewed literature. Announcing one's results before they are critically reviewed by peers can have disastrous impact on the credibility of both the scientist and his or her institute.

Educational Outreach

As science becomes more complex and difficult to comprehend, the need to educate the public actually increases. Science and engineering were once among the most respected professions in the United States. Our ability to improve our lives as well as achieve economic superiority over other nations depended on our scientific efforts. In recent years, however, the public image of science and scientists has diminished, and often we and our science are misunderstood or ignored altogether. Consider, for example, that one-quarter of Americans think that the Sun revolves around the earth, and nearly half of us do not believe in evolution (Levine, 1990). In large part this inability to comprehend science is because it is based upon additive experience and because it often involves mathematics (Wolpert, 1993), which is a particularly troublesome area for many Americans.

Coupled with poor scientific literacy is the increased need to communicate environmental science to the public. There are two clear reasons for this, one pragmatic and one more idealistic. The pragmatic reason is fairly obvious—the public ultimately pays for much of our research. They are our patrons, as it were. If we cannot communicate effectively to our patrons exactly what we do, we run the risk of losing their support. At the same time, there are important concepts, such as global warming, that we need to communicate to the general public. When a congressman states (as one did in 1995) that global warming is a myth and that the ozone hole is not expanding, does that not partly reflect our inability to present facts in a comprehensible manner? Should we not assume some responsibility for such a lack of comprehension?

On the more altruistic side, environmental science is probably the most practical way of teaching science. While radiation energy, wave theory, and biochemistry are concepts and fields that can confuse or bore the public, the coastal environment involves concepts that can be put into the context of the environment around them. Tides, waves, temperature, geology, and biology all become more easy to comprehend when the student can relate these concepts to something he or she can see or touch. What is more, the student immediately can see the practical application of such science; for instance, understanding the forces and impact of coastal erosion by seeing undercut houses falling into the sea.

Thus an appreciation for both science (in general) and the environment can be gained by exposure to coastal marine science, no matter if the classroom is a pond, an estuary, or the coast. But scientific education goes far beyond simply instilling an understanding of the environment and the processes that affect it. As Rutherford and Ahlgren (1990) point out, students also can learn the concepts of the working hypothesis, patterns of change, constancy, as well as mathematics and statistics. The trick, of course, is to make the exposure memorable, preferably one in which the student participates. Practical experience is often best. Learning about the biology of a mollusc from a textbook or a computer is probably less effective than actually holding a scallop in the hand, looking at its parts under a microscope, and studying its behavior in an aquarium.

A few years ago members of the Estuarine Research Federation and the National Marine Educators Association defined the following as the five most important concepts of estuarine education (after Spence *et al.,* 1990):

- mass balance of materials in estuaries with regard to both external and internal sources;
- watershed development and its consequences;
- estuaries as nurseries and spawning grounds;
- hydrodynamics of estuaries, particularly with respect to fresh- and salt-water exchanges; and
- physical-chemical-biological relationships in estuaries.

Note that each concept involves scientific principles that ultimately have practical implications and that these concepts do not differ greatly from those stated by the NRC (1994) (see above).

The goal in educational outreach should be to educate the general public, not to nurture future oceanographers and marine scientists. Some of the more motivated students might go on to major in science in college, perhaps even marine science, but the vast majority will ultimately benefit by becoming part of the educated public. Increasingly, educators are learning that this process cannot begin too early. Middle school students can understand science more easily if they have been exposed to interesting and comprehensible concepts in elementary school. Waiting until high school is probably too late for most students.

The number of k–12 outreach programs is increasing steadily. The North Carolina Museum of Life and Science, for instance, has issued a number of pamphlets that address "Sharing Science with Children," one dealing with the linking of scientists and engineers with students, and another with a survival guide for scientists and engineers. Our "Bay Team" program at the College of William and Mary is targeted at educating thousands of Virginia students annually. Even NOAA has directed considerable effort to educational outreach by its scientists and engineers (Baker *et al.,* 1993).

In some ways, educational outreach becomes more problematic when it involves adult education. Take, for example, primary school teachers who need a primer course in marine science that can be used for graduate credits required to maintain their teaching accreditation. One probably cannot teach these teachers a graduate-level course in marine science, some of whom probably never took a science course in college; nor will many faculty members volunteer to teach a less challenging course. Even if the course can be identified and an instructor chosen, how can graduate credits be justified?

On the other hand, outreach for the general public can be mutually educational for both the "class" and the "teacher," particularly if the outreach activity leads to a future research or funding opportunity. The outreach activity may be as simple as a periodic open house or as detailed as a workshop for legislators. As mentioned above, periodic open houses for the news media might be particularly useful, if not totally altruistic. As S. E. Cozzens (unpub. manuscript) states, "The sign of success is how broad a set of community leaders and citizens would be willing to testify convincingly on behalf of the university at state budget hearings, as well as the level of constituent compliments state legislators receive on the state's higher educational services."

In setting up or expanding such educational outreach programs, of course, one continually needs to ask many of the same basic management questions asked with respect to advisory services: What is the target group, who is the teacher, what is the duration of the event, what is the minimum, optimum, and maximum number of "students" we can serve, and how can we evaluate our impact on these students? Are there ways in which the educational outreach can be streamlined by using electronic technology, or does this simply detract from personal contact? Unless educational outreach is approached in a businesslike manner, it runs the risk of becoming an almost endless time-sink for faculty and staff, one that could disrupt the laboratory's other activities.

As for marine advisory services, one solution is to designate one or more persons specifically trained in educational outreach. This, however, might consume funds that could be used in other ways. An active docent program is an alternative solution that could ease the need for hands-on involvement by laboratory personnel.

Graduate Education and Public Outreach

Between the 1960s and the 1980s many students graduating with PhDs in the marine sciences were almost assured jobs in research and academia, initially in hard-money positions as colleges and universities began adding marine science to their curricula, and then in soft-money positions as new teaching opportunities decreased and research opportunities expanded. As recently as a few years ago, NSF talked about the need for a future generation of scientists both to staff new positions and to replace older scientists as they retire.

As we now know, the explosive demand for scientists in academia has not materialized. Nationally, more than half of the graduates with PhDs find work in non-academic fields (NAS, 1995), and in marine sciences this fraction may be greater. Data for U.S. marine laboratories are incomplete, but between 1991 and 1993 the number of academic faculty at the JOI+5 oceanographic institutions (Joint Oceanographic Institutions—defined as those schools and institutions with open-ocean research capabilities) remained essentially constant (Nowell, 1993). In 1993, moreover, only one third of the JOI+5 faculty were older than 50 and less than 10 percent older than 60, meaning that it might be at least 10 years before there is a significant number of faculty openings. Increasingly, therefore, we must look to other non-academic positions for our graduate students, not only those with masters degrees but also those with doctorates. One path, of course, is post-doctoral fellowships and non-tenurable faculty positions; but post-doctoral positions provide only a few years of respite from the real world, and surviving on soft money is an increasingly perilous existence in the flat-budget world of marine research (Wunsch, 1993).

The basic goal for all educators is to prepare students with optimal marketable skills, and successful marketing depends on considering new areas of employment. For marine scientists this means that applied research and public outreach must be considered viable alternatives to university teaching and research. Whether this involves education at the lower levels (*e.g.,* junior colleges or secondary schools), advisory work or joining profit-seeking organizations, the emerging markets in marine science appear to be in non-traditional areas.

This change in career opportunities in marine science is reflected in student interest in our graduate program at William and Mary. Between 1988 and 1994 applications increased 400 percent, and interest in management fields, such as environmental science and resource management, increased at an even greater rate. In comparison, between 1988 and 1993, applications to the JOI+5 schools increased by only about 60 percent (Nowell, 1993).

With the job market evolving into non-academic areas, graduate students can profit greatly from actively participating in public outreach programs. Solving concrete, real-world problems and needs can yield valuable experience that might lead to increased employment options. Working with advisory services, for example, can illustrate many of the problems that relate scientific knowledge to management issues, whereas education outreach can demonstrate effective ways to market scientific ideas to a non-scientific public.

Not only do we need to make outreach and advisory options more accessible to our graduate students, we also need to be more proactive by offering a wider variety of non-traditional graduate (and undergraduate) courses. As recently as a few years ago, graduate students at most schools were urged to take only basic science courses. The reality of today and probably for the foreseeable future, however, is that students, particularly those interested in coastal environments, will need training in managerial skills, including courses in business administration, economics, and law. My friend John Farrington tells of several physical oceanography graduate students in the MIT-WHOI joint program who recently took a course in industrial marketing, something that probably would not have happened a few years ago. Interestingly, the students requested the course—not their advisors. As pointed out by the National Academy of Sciences (1995), however, offering a greater range of academic options should not mean that other necessary courses are dropped or that the time to completion of degree is compromised.

Faculty Participation and Evaluation

As Land-Grant colleges and universities explore ways to increase their outreach to external constituencies, some incentives are needed for those faculty involved in outreach activities. A NASULGC (National Association of State Universities and Land-Grant Colleges) Newsline article (July 1994, p. 6–7) outlines the steps being taken by four universities (Clemson, Minnesota, Oregon State and Michigan State):

- —Create incentives to encourage faculty participation in outreach;
- —All extension faculty should be affiliated with an academic home (Oregon State);
- —Student participation in research or outreach will become a degree requirement (Clemson).

Sea Grant Marine Advisory Services (SGMAS) often include extension agents whose faces and names are unfamiliar to the academic or research faculty. SGMAS programs always reside within universities, but rarely are they part of important decision-making processes, and thus may be considered peripheral (UNC, 1992). As a result, professional advancement and career ladders for SGMAS personnel can be ill-defined or non-existent (UNC, 1992). To remedy these problems, the UNC report makes the following recommendations:

- —Outreach programs should be identified as an integral activity of the university;
- —Administrative and programmatic decisions regarding SGMAS activities should be integrated within the university system to facilitate the setting of policies, budgets and initiatives;
- —Agents and specialists should have clearly defined professional advancement opportunities;
- —Staff with advanced degrees should have the opportunity to obtain departmental affiliation and gain advancement within that department, including tenure.

The VIMS (1995) strategic plan states one goal as being, "(To) increase faculty awareness of educational outreach as an important area for faculty participation and service. Provide incentives for participation." But how outreach participation should be evaluated is open to debate. The problem is two-fold: one deals with the standards used in evaluating outreach, the other asks how outreach activity and results can be equated to teaching and research activities. The first problem is an absolute measure; the second is a relative measure. Teaching and research are relatively easy to evaluate: class enrollments, student evaluations, number of papers published, citation index, research grants, etc. But how does one evaluate the impact of teaching estuarine ecology to a middle school class for several hours, days or weeks? Because the rating standards are so qualitative, evaluations by definition will be fuzzy. Some of these problems are discussed in a recent book by Diamond and Adam (1993).

I remember about 20 years ago when a scientist at WHOI was warned to be less involved in the graduate education program and more involved in research; the scientist subsequently left WHOI. While this might not happen today, one wonders how various marine laboratories would respond to a faculty member who spends one day per week working with K–12 students or lecturing at retirement homes. Would the scientist be expected to do this in addition to normal office hours? Even if his involvement were condoned or even applauded, how would it be received by an external tenure review of that faculty member?

The traditional measure of a faculty member is the number of courses taught, students mentored, and (often primarily) peer-reviewed papers and citation indices. Not only are these considered accurate measures of productivity and impact, but they are relatively easy to quantify. It is much more difficult to evaluate the faculty member who spends considerable effort in outreach. How does one judge outreach effort and its impact? What are the terms of measure, can they be quantified, and how does one compare this against the more common measures of performance?

The problem becomes more complicated in a department containing faculty members who are essentially extension agents. In one version of a perfect world, all faculty members would participate equally in teaching, research, and outreach, and evaluations could be based on some integrated sum of the total effort. But in the real world, some faculty emphasize teaching and some research. While this division of labor nearly always creates controversy, most departments recognize the reality and (often) advantages of diverse activities. But the problems increase when one adds in extension service, which many still do not view as an academic activity, and certainly not one easily evaluated.

One obvious question is whether extension agents should have faculty appointments and be evaluated by the same standards applied to more traditional faculty members? At VIMS, where special emphasis is placed on outreach, there are two nested faculties. All faculty are members of the VIMS faculty, but only those directly involved in the educational program are members of the School of Marine Science (SMS) faculty. Appointment to the College of William and Mary, as well as tenure, resides within VIMS, and one can move on or off membership in SMS without losing the VIMS affiliation. As a result, many of our faculty more involved in advisory work and outreach belong within VIMS, but not SMS. While this may seem like a reasonable way to incorporate extension faculty into an academic department or school, the system is not without flaws. Membership within SMS is considered by most VIMS faculty as having a more prestigious status, leading some faculty involved primarily in extension work to seek membership in SMS. Yet if their annual teaching and research activities don't match SMS expectations, they can be removed from SMS.

Whatever the method of evaluation, no faculty can be evaluated fairly without some quantitative measure of his or her research effort and its net effect. The VIMS Strategic Plan (1995) states it rather simply, "(Advisory service) is . . . an integral part of overall scholarly expectations of faculty along with teaching and research, *but it is not a substitute for scholarship*" (Emphasis is mine). In other words, one cannot hide behind outreach. Extension agents, in fact, may need to spend more effort in becoming acquainted with new concepts and developments that may affect their areas of specialty. From this, scholarly research may ensue.

Conclusion

Marine science research and education appear to place increasing emphasis on nearshore processes and problems. At the same time, and almost certainly in large part because of this new emphasis, the need and demand for public outreach also are increasing. Coastal laboratories have an obligation to enhance their outreach efforts so that their research and educational activities are more directly applied to applied research and societal needs. The problems raised by this shift in direction, however, are not inconsequential; for example, laboratory organization, the management of outreach requests, gauging feedback, evaluating faculty performance, and so forth. But the payback in terms of increased research opportunities, increased job opportunities for graduate students, and the altruistic reward of communicating useful knowledge cannot be minimized.

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Coastal Laboratories and Environmental Management Agencies: Building Better Bridges

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Abstract. This essay will explore how coastal (*i.e.,* marine and Great Lakes) laboratories can be more effective in facilitating collaborations between research and management communities, thereby promoting sounder environmental policies and more effective environmental management.

Introduction

Research in support of management is a subject that I have been deeply concerned about for all of my professional life. It is an "ethic" that was instilled in me by my mentor, Donald W. Pritchard, and it was a guiding principle in the development of the Marine Sciences Research Center at the State University of New York at Stony Brook during the 20 years that I directed that Center. My most rewarding and my most frustrating moments as an academic science administrator were related to this mission.

In preparing this paper, I was reminded of a quote from the philosopher Nietzsche: "I have left the house of the scholars and I have slammed the door behind me. Too long I sat hungry at their table." Nietzsche describes better than I could, how I felt when I slammed the door behind me about a year ago. Viewing the table from a distance offers new perspectives. At times I long to be back at the table, but those times pass quickly.

The academic table is piled high with a vast array of "food": "food" that ranges from the traditional to the exotic. It is a table whose bounty comes from the investment of the public's money. The perception is that too little of the benefits of the bounty are shared—at least in a timely way—with the public that funded its creation. Some of the keepers of the table believe the food is much too sophisticated for common folk to appreciate. Much of the food on the table is perceived by the public to be only for the self-indulgent.

A few brave academic souls who gather regularly at the table to feast with their colleagues try to sneak some of the food out to feed the people outside the academic cathedral. For the most part, they are tolerated by their peers, but are generally considered to be lacking in "taste." They are often considered to be more suited to preparing fast foods for the public than exquisite meals for the gourmet. These deficiencies often are brought up at times of promotion and tenure. ". . . Many persons pride themselves on being pure theoreticians who believe that all research information is ultimately useful to society even though they might not have a clue at the moment as to how this usefulness will be manifested. Thus, while claiming to be 'pure,' they, at the same time, insist that their research funding be continued because of the ultimate applied value to society. What they scorn is helping society determine how to use the new information and even scorn those who attempt to do so on their behalf" (Cairns, 1993).

To push the table analogy further, we need to make the academic table less like an exclusive dining club for gourmets and more like a church supper or even a soup kitchen—a place where all who are hungry get fed in a timely way.

As with the world hunger problem, the primary problem with the academic table is not a shortage of food—although many academics would dispute that statement—it's an ineffective distribution system. Most academics are good in the kitchen, but ineffective on the distribution end—except in distributing food to members of their own guild who share their very specialized and idiosyncratic tastes.

Our challenge is to identify ways for universities, and particularly for marine laboratories to more effectively

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This paper was originally presented at a workshop titled *Roles of Coastal Laboratories in the Implementation of the Nation's Emerging Priorities for Research in the Coastal Zone.* The workshop, organized by the National Association of Marine Laboratories, was held in Sarasota, Florida, from 24 to 27 October 1995.

distribute the food on our table to serve society better. One of the primary classes of diners are environmental managers.

Because nearly all marine laboratories are associated with universities, I will comment briefly on the challenges facing universities. These challenges are particularly important to the future of marine laboratories because they are viewed by most mainstream academics as being ornamental and outside the university's core mission areas.

The Need for Change

If universities are to thrive, they must change. They should view this as an exciting opportunity, not a menacing challenge. Unfortunately most of the pressures for change continue to come from outside, rather than from inside universities. Most universities have failed to see the changing world around them as an opportunity to be seized. They see it as a threat and continue to hold on to an organizational model long outdated for all but a few of the most prestigious comprehensive research universities. Most universities appear to feel uncomfortable in seeking distinction through pursuit of distinctiveness. Being different in the academic world is uncomfortable for most academics.

While fundamental change in academia is needed, I am concerned that we could damage what academic institutions do best by insisting that they take on roles for which they are ill-suited. We have seen this happen in our public schools. I think there is another, more productive approach. It combines modest reform of colleges and universities with new mechanisms to engage them more productively in major societal issues.

Moreover, any substantial changes in university reward and governance systems will be slow in coming. I think most scientists would like to see their work applied. Most simply don't know how to do it. They are comfortable operating within the "tribe," but are uncomfortable moving outside the tribe. We have seen this in all kinds of technology transfer whether it is commercializing a breakthrough in the laboratory or in the application of new knowledge to solve an old problem.

In his book *Scholarship Reconsidered,* Ernest Boyer, President of the Carnegie Foundation, proposed a redefinition of scholarship to include four components:

- Discovering knowledge
- Integrating knowledge
- Communicating knowledge
- Applying knowledge (through professional service)

In the academic reward system the first—discovering knowledge—ranks number one by a country mile in considerations of promotion and tenure. The others should be given comparable weight. Boyer advocates the benefits of repeated cycling between theory and practice (Boyer, 1990). I can think of no better arena than the policy and management arena in which to practice the last three dimensions of scholarship—integration, communication, and application of knowledge—and no better institutions for application than marine laboratories.

In his 1990 address to the Association of American Colleges, Boyer referred to the institution that embraced these four dimensions of scholarship as the "New American College." He described them as institutions that take special pride in their capacity to connect thought to action; theory to practice. "This new American College would organize cross-disciplinary institutes around pressing social issues . . . Faculty members would build partnerships with practitioners who would, in turn, come to campus as lecturers and student advisors . . . The New American College, as a connected institution would be committed to improving, in a very intentional way, the human condition" (Boyer 1990). Boyer's choice of the word college, rather than university, may reflect that he was speaking to the Association of American Colleges, or it may indicate a belief that colleges are more willing to change than universities. The evidence suggests that they may be.

Marine laboratories might be able to accelerate the reform of university reward systems, at least at the "local" level. This is particularly true of those marine laboratories that have "college" status. If marine laboratories are going to be effective in developing and sustaining collaborations with management agencies, they need to place a clear, compelling and consistent emphasis on those qualities of leadership required of good collaborators. These adjustments should not be viewed as an example of lowering standards or of sacrificing quality, but rather of embracing flexibility in the criteria used to measure excellence in a changing world.

John Cairns argues persuasively for the need to expand the competencies of academics to include integration and synthesis, as well as greater breadth, so that they can communicate more effectively with managers, policy makers and the public (Cairns, 1993). At present these qualities are not highly regarded within academic circles. Most scientists and engineers are reductionists, because reductionism is the well-worn path to academic success.

I favor some broadening of the competencies of academics, and particularly of our students, so they will be equipped with the tools necessary for success in a society that demands a greater capacity for learning and doing. The portfolio model of Charles Handy might be a good one to follow. In this model, institutions not only are responsible for the "educating" of their students and for instilling a love of learning, but they are also places where each student can compile a portfolio of competencies. According to Handy, "Students should each be required to compile a bulging portfolio of certificates of competence or achievement" (Handy, 1994).

A different approach is needed to achieve the kinds of synthesis and integration that major environmental issues require and scream out for. The approach should exploit the depth of experts and achieve the necessary breadth through facilitated interactions—in forums—among experts from a range of disciplines. These forums should be in serial form so that major aquatic issues can be defined, clarified, and explored as they evolve. We are most in need of integration and synthesis efforts that not only have a significant vertical dimension, but considerable horizontal extent as well. If the environmental scientific arguments are separated from the social science and economic arguments, who will integrate them? For management and policy applications, integration must be done by teams of experts. This can be done most effectively if these individuals operate with the aid of skilled facilitators and the most modern information technology in 'holding environments,' a type of environment described in a later section.

One could talk at length about the many differences between the research and management subcultures. But I am more interested in finding ways for those two subcultures not only to communicate, not even only to cooperate, but to collaborate. Of the three "Cs," collaboration is by far the most powerful mechanism for effecting change.

Before we turn to a discussion of a model for facilitating collaborations of the research and management communities, let us briefly review these two subcultures.

Bridging the Academic and Management Subcultures

Much has been written about the differences between the management and research subcultures and how these differences impede communication, cooperation, and collaboration. In 1959 C. P. Snow wrote about the cultural differences between natural scientists and what he referred to as the "literary intellectuals" in a remarkable book titled *The Two Cultures and the Scientific Revolution.*

More recently, John Cairns stated, "If the book [C. P. Snow's] were written today, it could easily be titled *The 64 Cultures* or whatever number are present in today's comprehensive research university. In this case, the cultures are the disciplines, which retain their identity by various isolating mechanisms: (1) different requirements for rites of passage (*i.e.,* obtaining academic degrees); (2) physical isolation from other disciplines at academic institutions; (3) different "tribal" languages (an uncharitable person might call it "jargon") known only to the initiates; (4) different sources of funding; (5) different journals with each discipline having a select few crucial to tenure and promotion decisions, as well as for positive marketability (*i.e.,* a *must* to publish in these)" (Cairns, 1993).

The cultural differences between the research and management communities and among the academic disciplines are indeed real and should not be ignored. The incentives and rewards do differ in the two subcultures. Their styles of interaction and time scales for performance also differ, as do the tolerances of their constituents for failure and their demands for accountability.

Orbach points out another critical difference in the subcultures: "Generally the goal of policy-makers and constituencies is not to describe or explain, but rather to change and shape events and behavior. This is a critical difference between scientists and policy-makers and constituent groups: scientists produce data and information, while policy-makers produce change" (Orbach, 1991). Producing change is far more exciting than producing data and information. Given the changing views of the public on the priority for investing in basic research, academic scientists need, if not to become active agents of change, at least to collaborate with managers in applying advances in knowledge to major societal issues.

Orbach points out that "understanding . . . is not enough. Given that the subcultures share common goals—well-informed, well-documented, participatory public policy with respect to the coastal and marine environment—someone must build the bridge between the subcultures. Mutual goals, mutual respect—these are the basis of the cultural integration of scientists and policy-makers" (Orbach, 1991).

These bridges are critical, and they are much too important to institutional success to be left to a few individuals on an *ad hoc* basis. The leaders of the institutions—the marine laboratories in this case—must be the architects of these bridges. Each marine laboratory needs multiple bridges.

The cultural anthropology literature is rich with examples of how cultures that are isolated from each other for sustained periods—even though the physical separations may be small—develop myths about each other. Typically each culture ascribes undesirable characteristics to the other; characteristics that strengthen the forces of isolation. For example, often each may view itself as peace-loving and the other as war-like. It is only when members of the two cultures spend time in each others' "tribe" that these stereotypes are broken down. After working closely on many occasions with numerous members of both the research and management subcultures, I am convinced that this same model of myth-building is at work. The myths exaggerate the distinguishing characteristics and motives of the two subcultures.

The most effective way of overcoming cultural barriers is to break them down. As I have already stated, the best way to learn about another culture is to live and work in it. As a young graduate student in search of a meaningful career, I flirted briefly with psychology while at Harvard.

I particularly remember one course on cognitive psychology taught by the famous psychologist, Professor Jerome Bruner. He said "One can act oneself into a way of believing as readily as one can believe oneself into a way of acting." It is time to act.

We have seen the differences between the management and academic subcultures. They are distinct and distinctive subcultures, but the inhibiting effects of the differences on the potential for collaboration have been greatly exaggerated. Let's examine the similarities of the two cultures within which these subcultures exist. They provide the basis for developing models for collaboration.

Move Over Oscar: We All Live in a Garbage Can

Although the research and management subcultures may have important differences, they both operate under the same basic organizational model—the "garbage can" model. The "garbage can" model of organizations was developed originally for universities by Michael Cohen *et al.* and extended to government by John Kingdon. I will first briefly describe the model and how it relates to the policy-making process and then examine how a properly configured and managed "holding environment" that brings together key people from the two subcultures can exploit the peculiar qualities of the organizational model they share.

The garbage can model characterizes organized anarchies. According to Cohen *et al.*, organized anarchies have three general characteristics: (1) problematic preferences, (2) unclear technology, and (3) fluid participation. Organized anarchies are loose collections of ideas rather than coherent structures. Their members discover their preferences through action more than by taking actions on the basis of preferences. The state of their "technology" is unclear in that members of an organized anarchy do not understand the organization's total set of processes very well. They have very good knowledge about their local section of the garbage can, but little knowledge about what is happening in the rest of the can. And, the participation of members is fluid. They drift in and out of decision making. Those who show up at a committee meeting, or for a vote, are very important in determining the outcomes (Cohen *et al.,* 1972).

The outcomes of an organization with these characteristics are a function of the mix of garbage in the can and how the garbage is processed. According to Cohen *et al.*, four streams run through the university garbage: problems, solutions, participants, and opportunities. Cohen describes the university as ". . . a collection of choices looking for problems, issues and feelings looking for decision situations in which they might be aired, solutions looking for issues to which they might be the answer, and decision makers looking for work" (Cohen *et al.,* 1972). This certainly sounds like the university I used to work for.

In Kingdon's adaptation of the garbage can model to government, he identifies three streams running through that garbage can: problems, policies, and politics. According to Kingdon, most of the time the streams run independently, each with its own watershed and stream channel, each developing and flowing according to its own dynamics and roles. At critical junctures however, the streams converge, and the greatest policy changes grow out of the coupling of problems, policy proposals, and politics (Kingdon, 1984).

If our objective for marine laboratories is that they should play a role in influencing the development of policies that have a sound scientific basis, we should identify ways to choreograph these streams, causing them to intersect. We should work with managers to change their flows and their courses. Before examining a mechanism to do this, let's turn quickly to the policy making process.

According to Kingdon, policy making is a process that includes at least the following steps: (1) setting the agenda, (2) identifying and assessing policy alternatives from which a choice can be made, (3) making an authoritative selection among the alternatives, as in a legislative vote or a presidential or gubernatorial decision, and (4) implementing the decision—the policy. Only the first needs any clarification for our purposes. Kingdon defines the agenda as "the list of subjects or problems to which government officials, and people outside of government closely associated with those officials, are paying some serious attention at any given time" (Kingdon, 1984). If an issue isn't on the agenda, it doesn't get noticed.

According to Kingdon, the groups that shape public policy include elected officials and their appointees, career bureaucrats, non-governmental organizations (NGOs), and academic institutions. Kingdon points out that all of these groups have important roles to play in shaping policy, but that it is elected officials and their appointees who are most important in setting the agenda.

While marine laboratories may be only bit players in setting the agenda, they can be major players in affecting policy choices by identifying and evaluating alternatives. And, scientists have been known to get items on the agenda. Three examples are global climate change, the ozone hole, and tributyl tin.

Next we examine an institutional mechanism that will facilitate positive interactions between the research and management communities with the objective of improving environmental decision making.

Holding Environments

Marine laboratories can play important roles in the technology transfer of scientific knowledge to management applications. This requires institutional mechanisms that facilitate the technology transfer process. "Facilitate" is a wonderful, powerful word. It is a verb. It means "to make easier." Facilitation is an activity that is undervalued in most of academia, and in some marine laboratories. Making science accessible to the public is to facilitate the public support of science—an endeavour of immediate importance. Making the latest advances in science accessible to managers and policy makers, is to facilitate technology transfer to management. This also is crucial at a time when support for basic research is seen by many as a form of "welfare for the elite." Facilitation should be a priority of marine laboratories. The mechanisms deserve special attention.

Each marine laboratory should set up the institutional equivalent of what Ronald Heifetz calls a "holding environment" in his wonderful new book *Leadership Without Easy Answers.* A holding environment consists of any relationship in which one party has the power to hold the attention of another party and facilitate adaptive work. The term "holding environment" has its roots in psychoanalysis to describe the relationship of the therapist to the patient. Heifetz extended the term beyond parental and therapeutic relationships to anyone in a leadership position. I am convinced that the concept can be extended even further to include institutions. Adaptive work is defined by Heifetz (1994) as ". . . the learning required to address conflicts in the values people hold, or to diminish the gap between the values people stand for and the reality they face." Although it was only recently that I became aware of the concept of a "holding environment," we had created numerous examples of functionally equivalent activities at Stony Brook's Marine Sciences Research Center. They served us well through a series of budget crises over two decades.

At the New England Aquarium we have explored this concept with Professor Heifetz and have created the "Aquatic Forum" as our "holding environment." It is intended to be a safe environment in which major aquatic issues can be explored and in which the risk of failure is low. The goals are to clarify major aquatic issues, to reframe them, to state them richly in all their dimensions, and to identify the range of alternative strategies for dealing with them. These activities are carried out in public venues where representatives of all the key stakeholder groups are represented. Having agreed on the issue and on the approaches for dealing with it, a different meeting mode is used. The forum goes into executive sessions in which all the fields of expertise needed are represented along with a few "amateurs" to evaluate the alternatives in all their dimensions. As Erwin Cargaff observed: "Where expertise prevails, wisdom vanishes."

The concept of a "holding environment" is compatible with influencing policy and management practices at a variety of levels. Some of the most important collaborations probably are with mid-level managers with line authority in state management agencies. As Robert Reich has observed ". . . higher-level public managers are likely to have significant discretion over many of the problems they pursue, solutions they devise, and strategies they choose for implementing such solutions" (Reich, 1988). By keeping the latest research findings and management challenges bubbling together in "holding environments," marine laboratories can have a major influence on management policies and practices at the local, state, and regional levels. This influence should not be underestimated. It may not happen as often as it should, but it does happen more often than most scientists realize. It often is done quietly by one, or a few, scientists working closely over sustained periods with colleagues in management agencies.

These "holding environments" can become forums for adaptive management. Because trust is high, risk is low, and many management decisions can be treated as experiments that are subject to change as understanding increases or social priorities shift. The primary emphasis in the kind of "holding environment" I am proposing must be on using the knowledge we have and pushing it as far as it will take us. If the forums are seen as platforms designed to secure funding for more research, they will fail. The need for more research will inevitably be a result, but it must emerge as a priority of the managers. As the needs are identified and proposals developed, scientists and managers should be teamed-up from the outset. Members of the forum should be kept informed, and one criterion of a successful research project or program should be the extent to which it affected management policies and practices.

The "holding environment" has other advantages. It provides the level of protection needed for young professors. In the policy arena, time scales are long, pathways convoluted, and the flows of information dispersive and turbulent; the process of major policy changes can easily take a decade, or more. Policy making is not a rational decision making process. Too few scientists realize, or at least accept, this condition of the political process. One approaches it as if it were rational at his or her own peril. In the end, the original source of a policy idea is often difficult to identify. One should not stake one's future promotion on it. And directors of marine laboratories should not stake the careers of their young faculty on success in the policy arena.

Those in the "holding environment" can take the leadership by carrying out many of the functions that would characterize a rational policy-making process. They can formulate a clear, rich statement of the issue; a statement of goals; and an identification and analysis of alternatives. Through appropriate "holding environments," marine laboratories can focus sustained attention on major environmental issues and nurture collaborations of scientists, engineers and other scholars, managers, and policy makers to exploit advances in knowledge in the development of sound environmental policies and practices. Through this vehicle, the flow and the courses of at least the first two of the three streams—problems, policies, and politics—that run through the political garbage can, can be influenced. When all three are coupled, the greatest agenda changes occur, and the policy making process is triggered. Even when only two of the streams come together, things happen. As a result, the times of intersections of the separate streams are critical for those who want to influence policy: a problem is recognized, a solution is available, the political climate makes the time right for change, and the constraints do not prohibit action. A properly structured and orchestrated "holding environment" can facilitate change, but it requires a great deal of time, thought, energy and creativity.

The collaborations nurtured in a "holding environment" increase the likelihood that, when a policy window opens, a rational policy will be mature and will be floated through the opening. Because policy windows open quickly and often don't stay open long, "holding environments" must be on-going. Environmental advocacy groups and business groups learned long ago that there is a long process of softening up the political system for major policy changes. They work the political crowds with persistence and skill, waiting for the right moment. Marine laboratories have not, and should not, become partisan, but they must become more sophisticated politically if they want to increase the likelihood that the best science will be considered. The proper levels of institutional and individual protection are provided by institutional mechanisms that require low maintenance, but are effective in bringing the latest advances in science and technology to the "market place" of policy makers throughout the ebb and flood of public opinion.

On occasion, scientists also need to be reminded that science is only a tool for achieving societal goals and objectives. As Lewis Wolpert observes in *The Unnatural Nature of Science,* ". . . it would be a great folly to entrust decisions about how to use science to scientists or to any other group of experts . . . It is the politicians, lawyers, philosophers and finally all citizens who have to decide what sort of society we will live in" (Wolpert, 1993). Wolpert reminds us of Tolstoy's admonition "Science does not tell us how to live; it has nothing to contribute on moral grounds." Nevertheless, once society has selected its environmental goals and objectives, once it has decided how it wants to live, we should use the best science and technology to achieve those ends. At the very least, the policies that are implemented should not be orthogonal to good science.

"Holding environments" can provide another important service. They can be sites for the development of consensus among scientists on the scientific implications of major environmental issues. The power of scientific consensus is impressive if it involves a significant fraction of the most widely respected members of the scientific community appropriately scaled to the problem area, and if a few "wild cards" are added from outside for added credibility. Two cases in which the power of scientific consensus has been unambiguously demonstrated are Chesapeake Bay hypoxia and an environmental standard for monitoring the effects of fresh water diversion from the San Francisco Bay estuary.

Closing Observations

Ausubel and Steele describe the 'flattening' of the earth sciences as a trend toward larger, multi-investigator studies in which the scientists are drawn from an increasingly diverse set of institutions, nationally and internationally. The trend they describe is a consequence of globalization, a process that is occurring at an ever increasing pace. The driving force behind globalization is to create value added by brokering the most competitive combinations of resources of all kinds, including human capital, wherever those resources occur. The end result may be a leveling of universities and the larger oceanographic institutions. Its impacts on most coastal marine laboratories will be much less and will lag (Ausubel and Steele, 1993).

Many of us, over the years, have encouraged our faculty members to emulate their open ocean colleagues, building national and international teams with which to attack exciting and important coastal problems wherever they are most clearly manifested. Although team-building has affected investigations in open coastal waters, it has not been the dominant model in nearshore studies. There are exceptions, of course, and there are many examples of collaborations involving scientists from neighboring states who share a coastal waterbody.

The phenomenon Ausubel and Steele describe may make collaborations among scientists, managers, and policy-makers more difficult. Managers and policy makers need access to "groups" of scientists that have the collective breadth and depth to inform important management and policy decisions. Typically, they gain this access from one, or a few, individuals within organizations who have demonstrated a receptiveness to collaborate and a generosity with their time and ideas.

Michael Connor has pointed out that "commitment is maintained, re-affirmed, and nurtured by strong communication among scientists, managers, and the public. The first requirement is an easy point of entry for scientists and managers into each other's domain" (Connor, 1995). He also points out the value of having this entry point high in the organization. "This demonstrates the institutional acceptance (and more importantly encouragement and reward) for conducting science in support of policy and the follow-through to ensure that commitments are met" (Connor, 1995).

To the extent that the flattening described by Ausubel and Steele is manifested in the coastal research community, it will have an additional impact on those coastal marine laboratories that are state-supported. As scientists in these institutions spend more of their time working with colleagues from other states and other countries on problems in other regions, there will be greater urgency to demonstrate accountability and responsiveness to state problems if their core funding is to be sustained. Few state legislators I have met place as high a value on pushing back the frontiers of fundamental knowledge as they do on applying knowledge to improve the environment and the quality of life of those they represent.

"Research institutions, as places with laboratories, workshops, and offices will always be necessary . . . But will these places retain their individual styles of operation, their intellectual diversity, and an appropriate share of power? It seems unlikely that they can return to an earlier state" (Ausubel and Steele, 1993). As one who directed a marine laboratory for 20 years, I think the new world is filled with opportunities for marine laboratories to retain their intellectual diversity and an appropriate share of power, but only if they retain their "individual styles of operation." Those styles, however, may be quite different from the styles that have served them well in the past. Coastal marine laboratories must constantly search for new styles of operation that will make them distinctive. For most coastal marine laboratories, this will mean being much more creative in forming collaborations with the management and policy communities. The best mechanism I know of is the equivalent of a "holding environment."

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Networking the Infrastructure of Coastal Laboratories

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Abstract. How might the coastal laboratories organize to become a truly functional network? In answering this question we must first identify those elements of infrastructure, including facilities, services, and programs, that support the academic, research, and public service missions of coastal laboratories. Then we must identify mechanisms that will enable, where appropriate, the sharing of these infrastructural elements by the coastal labs and their user constituents. In this essay I also outline the siting and structuring of coastal facilities in the past, trusting that history will inform our efforts to meet current and future challenges and opportunities in coastal zone research. In the end I propose some specific activities at coastal labs that seem ripe for networking.

Introduction: Definitions and Presumptions

Let me first define "infrastructure" very broadly to include not only the physical facilities, instrumentation, and associated support technologies, but also their human resources (expertise) and functional organization. By "functional organization" I mean how a laboratory prioritizes, organizes, and supplies support services to its unique blend of scientific, educational, and outreach programs.

Let me next define "networking" as optimizing the shared use of infrastructure by the broad community of geographically dispersed coastal labs and their parent organizations. These parent organizations are often major universities or consortia, and they commonly mandate educational missions to their coastal laboratories. State and federal agencies and private corporations may also have missions involving research, monitoring, or public outreach at coastal facilities. Thus the infrastructure of a coastal lab supports some mixture of these activities, as is discussed in other papers derived from this workshop (*i.e.,* Feller and Karl, 1996; Milliman, 1996; Schubel, 1996).

A basic presumption is that 'the whole is greater than the sum of its parts'. The local mission of most individual labs could be enhanced, at least to some degree, if the resources of all the coastal labs were networked and shared. We also expect that the community of coastal laboratories, once they were working effectively together, would play novel roles in research, education, and public service and not only at the local level but at regional, national, and even global levels.

Historical Roots and Characteristics of Coastal Laboratories

Coastal labs are characterized not by their diverse infrastructures or programs, but by their sense of place. Historically, coastal stations were established to provide scientists and their students with access to particular habitats. The location of the station was rooted in an appreciation for a particular coastal environment: the rocky shores of New England or the Pacific Northwest; the panorama of salt marshes of the southeast or the Gulf of Mexico; the estuarine "coastal seas" of the eastern seaboard (*e.g.,* Buzzards Bay, Narragansett Bay, Long Island Sound, Delaware Bay, Chesapeake Bay, North Carolina's Pamlico, Neuse, and Albermale Sounds, and Biscayne Bay); the ocean-dominated coastal environments of the west coast (including Puget Sound, San Francisco Bay, and Monterey Bay); the coral reefs of Florida and the Caribbean; and later even the "urbanized estuaries." Some few coastal labs looked outward, making the oceans the focus of their research. But wherever located, the names and histories

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Abbreviations: CBL, Chesapeake Biological Laboratory; CEES, Center for Environmental and Estuarine Studies; VIMS, Virginia Institute of Marine Science.

This paper was originally presented at a workshop titled *Roles of Coastal Laboratories in the Implementation of the Nation's Emerging Priorities for Research in the Coastal Zone.* The workshop, organized by the National Association of Marine Laboratories, was held in Sarasota, Florida, from 24 to 27 October 1995.

of the nation's laboratories are redolent with this good "sense of place."

Already at the turn of the century, there was a debate as to whether there should be a concentration of marine research at a few national facilities. In his personal correspondence, Dr. Reginald Truitt, later the founder of the Chesapeake Biological Laboratory (CBL), argued for the formation of stations across the coastal geographic landscape "to insure access for studying and gaining an appreciation for the biology and resources of the marine environment." Several widely distributed field stations would provide a diversity of research sites, it was thought, and would not only address the interests of scientists, but also those of the local community.

Thus the local coastal habitat is the psychological grounding for each coastal lab. It is within that particular place—whether characterized by rocky shore, sand flats, marshes, coral reefs, kelp beds, or coastal upwelling currents—that observations led to specific research questions, and 'on site' research led to knowledge about the biology of marine organisms and emerging theory about the varying role different environmental factors play in regulating coastal systems. It is within that particular place that future science teachers and researchers would be exposed "hands on" to the wonders of the sea. It is within that particular place that a diversity of research, educational, and outreach missions of coastal stations would be initiated, evolve, and nuture a diversity of intellectual enrichment and practical approaches to understanding and appreciating the land-sea margin. It is within that particular place that various infrastructures—both facilities and interaction with the larger scientific community—would evolve. These infrastructures might not be unique but would certainly characterize coastal labs.

Given this geographical and psychological diversity of coastal labs, are there common threads that characterize the missions and thus the supporting infrastructures? Are there common problems and tensions that characterize carrying out these missions at sites usually distant from and thus without easy access to the support systems provided at a parent organization and in a more metropolitan area? In an important sense the answer to these questions will frame any discussion of what and how networking coastal labs might strengthen individual needs, open new vistas for collegial opportunities, and contribute to the broader aspirations of the science community in coastal zone research.

Infrastructure of early coastal laboratories

Coastal stations provide access to coastal habitats and local organisms and the on-site facilities to support the study of marine life and its environment. These are the common features of almost all coastal stations, and they have always been the fundamental shapers of coastal lab infrastructure.

When most early coastal labs in the United States were founded, about 90–120 years ago, travel to the site, whether by rail, boat, or road, was usually time consuming and arduous. But scientists with academic ties could, with profit, take the whole summer off to conduct field or laboratory research. Moreover, the research could be coupled with teaching in courses for students or pre-college teachers. Thus, facilities for living—and work—became the backbone of early coastal laboratory infrastructure: dormitory and dining facilities, laboratory space and instrumentation for instruction and research and boats and seawater systems to collect and maintain organisms. And because a station might be far from its parent institution, access to scientific literature was necessary, so on-site libraries were established and often became sources of pride. Monographs and keys were indispensable for identifying local biota, as was an organized collection of local organisms. And a "database" of local environmental conditions was another legacy of the coastal laboratory. The variety of support services needed to sustain such an isolated "academic village" developed around this basic infrastructure: carpentry and mechanical shops and administration.

NSF-Sponsored Workshops on Field Stations

Three times during the last 15 years—in 1981, 1986, and 1995—the National Science Foundation (NSF) has sponsored workshops to assess the facilities needs of marine labs and field stations (NSF, 1981, 1986, 1995). The 1981 and 1986 workshops included a group of laboratory directors who were invited to evaluate the effectiveness of the NSF program on equipment and facilities for research at marine labs. The reports from these workshops were reviewed by a large number of additional lab directors. The most recent workshop (1995) was jointly organized for NSF by two associations: the Organization of Biological Field Stations (OBFS), comprising the directors of more than 145 field stations (mostly fresh water and terrestrial); and the National Association of Marine Laboratories (NAML), comprising directors of more than 90 coastal (marine and Great Lakes) laboratories. The directors from these organizations based their workshop report on a comprehensive assessment of the current state of research and education at field stations and marine laboratories. A comparison of these three workshop reports reveals how the changing mission of the laboratories has influenced perceived needs for infrastructure support and how access *to* laboratories has broadened to access *out from* and *among* laboratories.

The 1981 workshop report, "U.S. Marine Laboratories: A Plan for Modernization and Maintenance," focused on biological research laboratories and stressed their role as "repositories of knowledge" and their classical, time-honored policy of providing access to habitats, animals, and support facilities to a national, multi-institutional clientele. This open-door policy was seen to lead to a dilemma: that is, although these stations provide a valuable service to visiting scientists and are enhancing national scientific goals, their revenue—largely local—cannot support the additional infrastructure required by the visitors. So the financial shortfall caused a major infrastructural shortfall: reduced access to scientific instruments, central facilities, and services. Among the facilities specifically mentioned as needing infrastructure support were seawater systems, vessels up to 50 ft, libraries, electron microscopes, computers, radioisotope facilities, and electronic, machine, and photography shops. The report recommended that funds be provided to supply or enhance these facilities; and with respect to education, it recommended that the costs of transportation of students to courses at marine laboratories be subsidized.

The 1986 workshop, "Marine Biological Laboratories: Equipment and Facilities for Research," stressed the concept that these stations serve as "platforms for marine research." The report recognized the interdisciplinary nature of coastal research and noted that the missions of marine labs had broadened to include resource management and outreach education. Although the local roots were acknowledged, the place of marine labs in the developing perspective on the global environment was discussed. Moreover, the expanded role of marine labs in support of such experimental facilities as mesocosms was used to justify the need for increased funding for these institutions. The report did not consider any interaction and coordination among marine labs.

The 1995 workshop, "A New Horizon for Biological Field Stations and Marine Laboratories," asserted that the diversity of field settings is the most important attribute of these institutions, and stressed that providing direct access to local habitats is a crucial role. But the major thrust of this most recent workshop was the transfer of perspective and resource distribution from individual stations to a broader community of stations. For example, the report suggests that each station not only evaluate the vitality of its local biosphere, but also serve as a node in a formal, functional network of field stations and marine labs. The report bases its recommendation on dramatic effect of computerization and information access on the conduct of science, facilitating especially collaborations between geographically dispersed teams of scientists. By analogy, therefore, the stations and laboratories should begin to share data and ideas.

The importance of access to study sites and the availability of equipment and support facilities for on-site research training was also reiterated: "research is the vehicle through which students experience sound science." The report indicated that 80% of the institutions have an educational mission, and that housing related to that mission is the top new construction need.

Three points emerging from these three workshops bear consideration:

1. Some of the "village" services of yesterday have now become highly technical. The sophistication and transdisciplinary character of modern marine research (Morse and Nealson, 1996) has greatly increased the complexity of the infrastructure required at the nation's coastal laboratories. Even in the past 15 years, the research at many stations has expanded from biological field studies to experimental research requiring more sophisticated instrumentation, augmented technological support facilities, and new or renovated buildings to contain them (Feller and Karl, 1996). Moreover, the need for such core infrastructural elements as living facilities, laboratory facilities, library and information services, vessels and vehicles, and shops of various kinds has not diminished. The expansion of research approaches and the accelerated appearance of new technologies require that we examine new models of operation based on shared access to libraries and information services, effective use of costly instrumentation and support facilities, and regional availability of vessels.

2. Field access to local habitats is consistently viewed as the unique attribute of coastal laboratories. Yet marine research is, rightfully, evolving global perspectives. Moreover, the increasing tendency for the marine research community to organize resources, both intellectual and physical, around big science questions is diminishing the role of individual laboratories (Ausubel and Steele, 1993). The loss, however, of that rich and productive diversity of perspective and observation afforded by the coastal laboratories—that "sense of place"—would be unfortunate (Morse and Nealson, 1996). The solution to this dilemma may reside in new infrastructures that will help synthesize both the contributions of individual scientists and the local community within which they belong. The '95 workshop hit the mark when it focused on recommending the development of a communications networking of laboratories to improve the exchange of ideas and information. Such a network would foster the continued contribution by the coastal laboratories of their rich mosaic of talent and knowledge.

3. The infrastructural needs of research were the primary concerns at the three workshops; so we cannot be surprised that the needs for educational opportunities at coastal laboratories are mentioned only cursorily in the reports. Yet traditional models of station-based education were clearly assumed: the 1981 report identified the need to bring students to centralized courses at coastal laboratories; the 1985 report alluded to the development of

public awareness of coastal zone research; the 1995 report restated the importance of access to study sites and on-site research. But no exciting expanded educational roles—either in traditional academic programs or in outreach—was envisioned for the coastal labs. The expectation that research will be integrated with public outreach and with the traditional educational roles of the coastal laboratories (Milliman, 1996) means that infrastructural support, especially telecommunications and information access, must be rethought and designed.

Telecommunications infrastructure

The heart of the evolving design and networking of the infrastructure of coastal stations is the communications revolution. The introduction of fiber optics and other technologies in telecommunications has already transformed—and will surely transform again—how we conduct research, how we educate, how we garner public support for our science, and how we provide access from the coastal labs. Ausubel and Steele (1993) point out that a generation of scientists has, due to technological advances, grown up working closely with remote scientists via telecommunications. They argue that this kind of collaboration across institutional boundaries could lessen the traditional need for research institutions in diverse locations. They agree that some marine research institutions will always be necessary, but wonder whether they will retain their individual styles of operation. They foresee the evolution of an analog to the business world's virtual corporation: a blend of core facilities and services linked through a communications network to dispersed, decision-making functional units. (It should be noted that the authors are writing from the perspective of the large oceanographic institution; it might be argued that a stronger "sense of place" more characteristic of smaller coastal labs will provide deeper institutional roots.)

Ausubel and Steele (1993) propose that these changes provide "healthy ties between individuals and their institutions." Might not such networking also provide healthy ties among coastal laboratories by strengthening and expanding their individual and collective contributions to the national agenda in coastal zone research? The promise of communications technology lies, not just in scientist-to-scientist interactions (*e.g.,* telephone, facsimile, Internet, video meetings), but also in laboratory-to-laboratory interactions [*e.g.,* library and information services, research platforms (including vessels, boats, and remote sensing), instrumentation, educational programs]. In the latter case, it is infrastructural support that is shared, leading to the enhanced performance of all the participants.

Library and information services

Provision of some access to the scientific literature has always been considered an essential service at isolated coastal stations. The library operated jointly by the Marine Biological Laboratory (MBL) and the Woods Hole Oceanographic Institution (WHOI) is exemplary, for both historical perspectives and newly emerging ideas are available to scientists. But even the smallest coastal laboratories strive to collect essential academic resources. For example, the faculty at CBL, even in times of limited resources, has recognized the investment of library holdings and has consistently chosen to support increased library funding. But as library costs increase, we must share library services. Rapidly evolving communications technology already allows this networking. Most labs will want some combination of on-site materials and efficient yet cost-saving access to the greater services that large libraries offer. Most university-related laboratories have links to some of the library services offered by their parent institution. But we should also become involved with parent-institution libraries much earlier—even as they are developing the intercampus infrastructures that will allow access to their spectrum of library functions and services. We might also negotiate attractive contracts with libraries to supply access to more specialized library and information services.

Telecommunications technology in the on-site library also allows us to examine critically the suppositions that underlie acquisitions. Rising journal costs have led libraries to look hard at more efficient alternatives. Widdicombe (1994) has described criteria that, when applied, led to significant cost savings, and yet increased customer satisfaction through a combination of actual holdings and electronic access.

Boats and vessels

The restriction, in the 1981 workshop, of support to boats under 50 ft highlights the tension in the marine science community between support of open oceanic versus shallow-draft vessels that are useful and versatile in coastal habitats. Malone and Wright (1994) summarized part of an NSF-sponsored workshop on research vessels and gave a good introduction to the kinds of planning that must be done if research platforms that can be used in the coastal zone are to be available: *e.g.,* selecting the variety of platforms (fixed moorings, drifters, underwater vehicles, aircraft, satellites); designing for the broad spectrum of coastal habitats; identifying unique needs for long-term sampling; and recognizing common vessel requirements for particular regions. These issues are not within the purview of this paper, but effective networking in planning these facilities and considering the potential for sharing them will be crucial to the success of the marine science community. Just as NAML is composed of regional groups that often focus on more local concerns with a more local perspective, networking of coastal laboratories must encourage the formation of nested user groups that will serve regional needs.

Part of networking the use of the research platforms extends to the technical support and inter-institutional sharing of real-time data via remote sensing. The telecommunications revolution has freed remote sensing protocols from the bottleneck of data overhandling. An exciting project under way in the Chesapeake Bay, involving CEES, VIMS, and Old Dominion, in partnership with NOAA, is CBOS: the Chesapeake Bay Observing System. CBOS currently deploys two moored buoys (eventually five) throughout the Chesapeake Bay that send back real-time environmental data via radio telemetry on an ecosystem-wide scale. A guiding principle in developing CBOS was easy access to data for both scientists and the general public, and CBOS data is now available through the World Wide Web. Similar programs at Scripps, in Tampa Bay (PORT), and on the Great Lakes are the harbingers of future research tools with underpinnings in telecommunications technology that can be easily accessed.

Instrumentation

The inexorable inflation in the acquisition and maintenance costs of increasingly sophisticated instrumentation used in marine science makes a strong argument for networking many types of analytical services (Wunsch, 1993). Groups of individual coastal labs will need to support specific analytical centers for cost-effective quality data. This networking, however, must not be limited to fee for sample, but must also encourage the effective management of the talented and expert research scientists involved in developing and evaluating new instrumentation and protocols, intercalibration exercises, and estimating realistic needs for analytical services centers.

Educational programs

Coastal labs have interacted least in networking educational opportunities, whether aimed at the marine science community, or at those interested in outreach. This inertia is unfortunate because practical experience, in the field or in the laboratory, is at the heart of all science education, but is especially important for survival and success at coastal stations. We have seen an expansion and diversification of educational opportunities at coastal labs, from a wild variety of courses on all aspects levels marine life and exposure to field research, to outreach education, and promoting public awareness of the coastal zone. Yet there has been little sharing of resources or development of program partnerships.

One action that could enhance the success and soundness of academic programs at coastal stations would be for a group such as NAML (1) to acquire and keep current a central schedule of undergraduate and graduate course offerings and a file of dormitory availability at the coastal laboratories; and (2) to organize a workshop to evaluate how the coastal lab community could support course offerings that are designed for multi-institutional participation. A group such as NAML might also take the lead in using new telecommunications tools to evaluate educational experiences. The ASLO meeting in 1995 included a workshop on the use of emerging technologies in undergraduate aquatic science education (Weiler, 1995). The session topics of the workshop point to the potential of new teaching paradigms: classroom applications of marine and environmental remote sensing data; the use of environmental data on the Internet for undergraduate research, classroom simulation of a lotic and lentic ecosystem, multi-media for oceanography, interactive tools for teaching earth-system science, web access to El Niño-related information in real time. A particularly lively way to share educational resources in coastal science is through interactive video. The pioneering work of the Jason Project brought a practically inaccessible field site to the students. Aquaria should further develop partnerships with labs to enrich public awareness of coastal habitats.

Presently many of the NAML labs have begun to offer interactive video courses with their parent institutions. Some are exploring team-teaching in this medium, where faculty from several labs would participate to enhance the instructional expertise and habitat diversity of the course.

Some Final Considerations in Implementing a Network of Infrastructure

The design of a network of support services for coastal laboratories will rest, to some degree, upon the expansion of telecommunication technologies. Technology drives science. Technology, especially in telecommunications, will be an important driving force that will greatly influence infrastructure in most institutions. In response, we need to make the effort to understand that technology, to evaluate its potential to our academic world, to realize its limitations, to keep abreast of its rapidly changing capabilities, and to share that knowledge within the coastal lab network.

We must be flexible and open to change in such rapidly changing technologies; we must avoid what I call the cement-tank approach. In my early days of research in large-scale culture of marine organisms, I learned that choosing cement tanks of fixed dimensions stifled your "what if" musings about different configurations. We need to prudently choose the best technologies on the basis of compatibility and versatility for interconnection.

In all we do we need to balance networking uniformity with local diversity, that historically unique attribute of the coastal laboratory. Observations will be the underlying pinning of science regardless of the instrumentation and mathematical modeling that serves science so well. We still need the unique "looking glass" that each coastal lab provides to appreciate and understand the rich environmental mosaic where land meets sea.

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Thyroxine-Accelerated Larval Development in the Crown-of-Thorns Starfish, *Acanthaster planci*

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Dramatic population increases (outbreaks) of the coral-eating crown-of-thorns starfish, Acanthaster planci, *have resulted in damage to many Indo-Pacific coral reefs, especially on Australia's Great Barrier Reef. Research on factors that could affect* Acanthaster *distribution and abundance has included investigation of larval development, dispersal, settlement, and metamorphosis. For example, various substrates, particularly crustose coralline algae with bacterial surface films, induce* Acanthaster's *planktonic larvae to settle and metamorphose (1, 2), but little investigation has thus far been devoted to specific chemical substances that affect various phases of* Acanthaster *development. We report here that thyroxine, which accelerates development in three sea urchin species (3), also accelerates* Acanthaster's *developmental progress through larval stages and that this acceleration is concentration dependent. Our results suggest the need for research on possible roles of iodinated organic molecules in* Acanthaster *development* in situ, *and on the effects of additional substances known to accelerate the development of other echinoderm larvae.*

Acanthaster planci is one of several tropical, Indo-Pacific asteroids that prey on coral polyps (4), but unlike the others, *Acanthaster* has seriously damaged coral reefs during episodes of substantial numerical increase (outbreaks). The wave of *Acanthaster* outbreaks that began in the late 1970s, spread southward through Australia's Great Barrier Reef, and resulted in damage to many reefs is now ending in southern portions of the system (5), but numerical increases that could signal the beginning of a new set of outbreaks are being observed on some reefs to the north (6).

Acanthaster's life history includes a pelagic larval phase followed by settlement and metamorphosis on coral reefs. Several larval adaptations that could facilitate long-distance dispersal have recently been described (7–9). Recruitment of larvae into particular communities is a major determinant of local abundance. Settlement and metamorphosis, which are key steps in recruitment, are induced in the larvae of some marine invertebrate species by the presence of conspecific adults, thereby adding juveniles to monospecific aggregates (10–12). Other larvae respond, by extensively studied mechanisms, to molecules that signal the presence of potential food sources or other environmental factors favorable for juveniles (13–23). Various substrates induce the settling and metamorphosis of *Acanthaster* larvae (1), but certain crustose coralline algae are especially effective in this regard, and recent evidence suggests that bacteria on the surface of these algae are also involved in evoking the larval response (2). The neurotransmitter γ-aminobutyric acid (GABA) induces larval settling, metamorphosis, or both in various invertebrates (14, 17–22), as does excess KCl (24–26), but R. Olson found that neither GABA nor KCl elicited responses by *Acanthaster* larvae (cited in 27).

We examined responses of *Acanthaster* larvae to thyroxine, which accelerates adult rudiment development in sea urchin larvae (3). In one set of experiments we transferred samples containing larvae at various stages of development, but with most being at the mid-brachiolaria stage, to culture dishes containing artificial seawater with thyroxine added. After 26 h we found that development of the larvae exposed to thyroxine was significantly accelerated compared to controls (Fig. 1). We then added coral fragments with crustose coralline algae on their surfaces to control cultures and cultures exposed to two concentrations of thyroxine. The experiment was terminated 3 days later when all of the larvae in some of the culture dishes had settled. Coral fragments were removed so that all surfaces could be inspected microscopically and the percentages of settled larvae and juvenile starfish could

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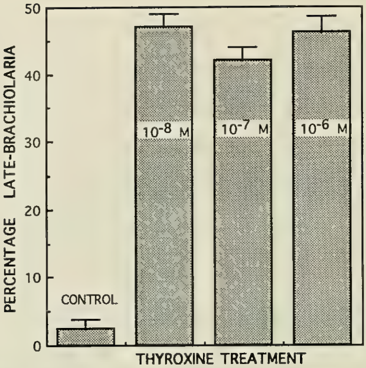

Figure 1. Mean ($n = 5$) percentages of late-brachiolaria-stage larvae, with standard errors, observed after 26 h in culture dishes containing thyroxine at several concentrations. Larvae reared by standard methods in 80-l cultures were transferred to glass dishes containing 80 ml of artificial seawater (Woods Hole MBL Formula). Transferred samples contained individuals at several larval stages, but most were at the mid-brachiolaria stage. Larval density was less than 1 larva per milliliter. Unicellular algae were supplied to control and thyroxine cultures at the same density provided in large batch cultures. All cultures were maintained in a 27°C constant temperature room. Developmental stages of swimming larvae were scored using criteria adapted from Birkeland and Lucas (27).

be determined. This handling necessitated termination of the experiment at that time. Thyroxine-treated larvae apparently remained somewhat ahead of the controls at the end of the experiment because more of them had settled or metamorphosed (Fig. 2). Because we do not know what would have happened to the control larvae if the cultures could have been maintained longer, we cannot conclude from these results that thyroxine treatment increases the percentage of settlement or metamorphosis, only that it accelerates larval development. These results also confirm that thyroxine-treated larvae can successfully complete metamorphosis.

To verify and more precisely quantify acceleration by thyroxine, we transferred early-brachiolaria-stage larvae taken from a highly synchronous culture to dishes containing artificial seawater and various thyroxine concentrations. After 2 days, we observed that thyroxine-treated larvae were longer than control larvae. The mean length of larvae treated with 10^{-7} M thyroxine was 1085.2 μm (SE = 20.55) and of control larvae was 894 μm (SE = 25.18). This difference was significant (Student's t-test, $P < 0.001$). Thyroxine-treated larvae also were developmentally accelerated, and the degree of acceleration depended upon thyroxine concentration (Fig. 3). This thyroxine-induced developmental acceleration resembles that found in three echinoid species (3). Thyroxine has been found to be much more effective than structurally similar iodinated compounds in accelerating sea urchin development, but the mechanisms by which thyroxine increases the rate of development in echinoderm larvae remain to be studied.

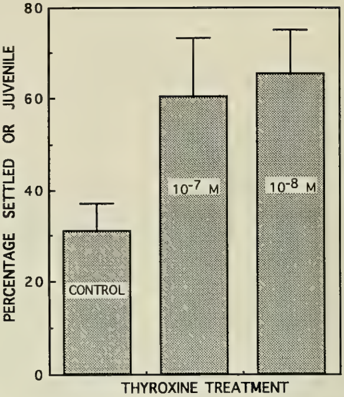

Figure 2. Mean percentages of settled (attached, metamorphosing) larvae and juvenile starfish, with standard errors. Larvae and juvenile starfish were tallied 3 days after three coral fragments with crustose coralline algae on their surfaces were added to each culture dish. All cultures were maintained in a 27°C constant temperature room.

Because any factors that accelerate larval development could increase survival by reducing time of exposure to predation or accident, our results suggest several other questions for future investigation. Are iodinated organic molecules or other development-accelerating substances present in the water around crustose coralline algae and do larvae show chemotactic responses to such substances? Do *Acanthaster* larvae respond to additional substances

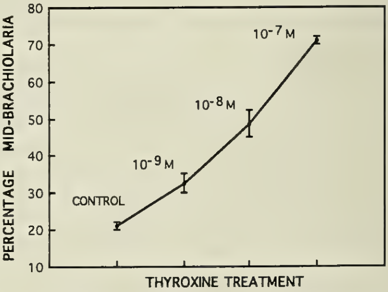

Figure 3. Mean ($n = 6$) percentages of mid-brachiolaria-stage larvae, with standard errors, observed after 2 days in culture dishes containing thyroxine at several concentrations. Methods as in Figure 1 except that larvae were uniformly at the early-brachiolaria stage when initially transferred to the culture dishes and that cultures were concentrated to a small volume of seawater and poured into the grooved troughs of a counting chamber for precise staging of larvae.

that accelerate development in other echinoderms (13–18, 23)? Might the relationship between *Acanthaster* juveniles and the coralline algae on which they settle resemble those of other coralline algae and the herbivores that graze them (28) such that release of factors influencing larval development could be adaptive for the algae?

Spangenberg has proposed that thyroxine, which has been found deposited in various structures including mineralized portions of several invertebrates, plays a role in regulating development in a cnidarian. She reported that experimentally supplied thyroxine induces strobilation and affects statolith development in the jellyfish *Aurelia* (29–31), and that thyroxine and two of its iodinated tyrosine precursors are produced in *Aurelia* just before strobilation and not at other times. Spangenberg's results together with recent observations of thyroxine-accelerated development in members of two echinoderm classes suggest that this signaling molecule, which has diverse effects on development in various chordate animals and acts as a powerful metabolic regulator in homeothermic vertebrates, might have an evolutionary lineage that extends through the developmental responses of the deuterostome ancestors of echinoderms and chordates to even more ancient roots.

Acknowledgments

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Reference: *Biol. Bull.* **190:** 302–312. (June, 1996)

Reversing the Life Cycle: Medusae Transforming into Polyps and Cell Transdifferentiation in *Turritopsis nutricula* (Cnidaria, Hydrozoa)

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Abstract. Organisms develop through a series of stages leading to sexually mature adults. In a few cases ontogeny reversal is possible, but it does not occur typically after the onset of sexual reproduction. All stages of the medusa *Turritopsis nutricula,* from newly liberated to fully mature individuals, can transform back into colonial hydroids, either directly or through a resting period, thus escaping death and achieving potential immortality. This is the first metazoan known to revert to a colonial, juvenile morph after having achieved sexual maturity in a solitary stage. Selective excision experiments show that the transformation of medusae into polyps occurs only if differentiated cells of the exumbrellar epidermis and part of the gastrovascular system are present, revealing a transformation potential unparalleled in the animal kingdom.

Introduction

Hydrozoan life cycles are among the most varied of the animal kingdom, with a vast array of different patterns (Boero and Bouillon, 1993). The basic hydroidomedusan life cycle proceeds from the adult medusa (a solitary stage), to the planula larva, to the polyp (the hydroid, an asexual and often colonial stage). Medusae are produced asexually by polyps. In most cases, the medusae have a limited life span, with a growth phase leading to sexual maturity and liberation of gametes, sometimes in multiple episodes of gamete production, followed by cell disintegration and organismic death. Ontogeny reversal, with direct transformation of isolated medusa buds into polyps, has been demonstrated to occur in *Podocoryne carnea* (Müller, 1913; Frey, 1968; Schmid, 1972), *Eleutheria dichotoma* (Hauenschild, 1956), *Cladonema* sp. and *Cladonema uchidai* (Kakinuma, 1969), and *Perarella schneideri* (Piraino, unpubl. obs.). These isolated medusa buds can revert to polyp structures only at the beginning of their development, completely losing this ability before they are liberated. Recently, however, a unique case of ontogeny reversal has been reported by Bavestrello *et al.* (1992), in which newly released, sexually immature medusae of *Turritopsis nutricula* McCrady, 1859, regressed, settled onto a substrate, and gave rise to stolons and hydroid colonies. The onset of sexual reproduction has been hypothesized to be the point of no return in the ontogenetic sequence of any living organism (Stearns, 1992). We evaluate this hypothesis by studying in the laboratory both the potential for and the triggers of transformation throughout the life span of *T. nutricula* medusae.

We have also investigated the cellular basis of this transformation process. The medusa is distinguished from the polyp, not only by a different shape and anatomical organization, but also by a completely different set of somatic cells in the umbrella. A layer of mononucleated, striated muscle cells lines the subumbrellar cavity, and specialized nerve rings and sensory organs (ocelli and statocysts) are present only in the medusa (Tardent, 1978; Mackie, 1980; Werner, 1984). Differences in patterns of protein expression between polyps and medusae were detected by Bally and Schmid (1988) and, more recently, RNA fingerprinting data confirmed these distinctions at

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the genomic level (Aerne *et al.*, in press). In addition, the epidermal muscle cells of the hydroid produce a chitinous covering (perisarc), but the naked exumbrellar, nonmuscle cells of the medusa do not. Thus, transformation of the medusa into a hydroid must involve substitution of cell types and tissue regeneration and reorganization. Both hydroids and medusae can regenerate missing tissues and organs, but regenerative potential is not solely associated with the proliferation and differentiation of cycling embryonic cells (interstitial or I-cells). Regeneration in medusae is more likely to be due to the remarkable transdifferentiation potential of fully differentiated cells, as demonstrated for tissues of the medusa of *Podocoryne carnea* (reviewed in Schmid, 1992). Transdifferentiation is defined as a change of commitment and gene expression of somatic, well-differentiated, noncycling cells to other cell types directly or through the return to a condition of undifferentiated cells. In the past, this process was called metaplasia or cell transformation (reviewed in Okada, 1991). The pioneering work by Wilson (1907) opened a long-lasting debate on the reversibility of cell commitment and functional stability. In recent years, the pluripotency of nuclei of differentiated cells and the activation of silent genes have been demonstrated through transplantation studies and DNA-binding proteins (Di Berardino *et al.*, 1984; Di Berardino, 1988; Topscott *et al.*, 1988; Lassar *et al.*, 1989). The processes of transdetermination of embryonic cells and transdifferentiation of specialized somatic cells, having strong ties with regeneration processes both in invertebrates and vertebrates, demonstrate that cell commitment and functional stability are not consequences of loss or irreversible inactivation of genes (reviewed by Okada, 1991; and by Schmid, 1992).

From the foregoing considerations, the transformation process of *T. nutricula* medusae can be explained, at the cellular level, by two alternative, but not mutually exclusive, hypotheses. First, all differentiated somatic cells of the medusa degenerate, and the production of polyp cells is initiated by a set of undifferentiated reserve cells that were not irreversibly committed. Second, differentiated cells of the medusa might transdifferentiate to produce the requisite new cell types.

Materials and Methods

Rearing

About 4000 medusae of *Turritopsis nutricula* were obtained from hydroid colonies collected in the Gulf of Naples (Western Mediterranean) from June to October, in 1993 and 1994. The medusae were reared in the laboratory at 20°C in groups of 100 individuals in 500-ml beakers, and fed every other day with newly hatched brine shrimp (*Artemia salina*) until gonad maturation and spawning. Slight water circulation was maintained by a gentle stream of air blowing across the surface of the water.

Immunohistochemistry

Whole medusae were anesthetized in $MgCl_2$ (3.5% [w/v] in seawater), and fixed in 4% paraformaldehyde (PFA; pH 7.2) in phosphate-buffered saline (PBS, 150 mM NaCl, 10 mM Na_2HPO_4; pH 7.4) for 10 min. Then the medusae were washed twice for 5 min in PBS containing 0.05% Triton + 0.05% NaN_3 (PBS/Triton) and rinsed in two changes of PBS. The histological and anatomical organization of the medusa of *T. nutricula* was investigated by incubating for 1 h at room temperature in 1:100 dilution of either (a) rabbit polyclonal antibodies raised against RFamide positive nerve cells (antisera kindly provided by the laboratory of Prof. C.J.P. Grimmelikhuijzen), or against human "$\beta1$"-integrin; or (b) mouse monoclonal antibodies raised against smooth muscle cells (termed sm-1) or against striated muscle cells (termed 93, Schuchert *et al.*, 1993) of *P. carnea.* All showed cross-reactivity with *T. nutricula* tissues. Subsequently, the medusae were washed in PBS (10 min, two times), and incubated in FITC-conjugated goat anti-rabbit IgG (Sigma) or anti-mouse IgG (Sigma) diluted 1:20 with PBS, for 30–60 min at room temperature. Finally, the medusae were washed in PBS and stained with 4,6-diamidino-2-phenylindole 2 HCl (DAPI, 0.05 mg/ml; Serva) in PBS for 1 min, washed with PBS, mounted in 10:1 glycerine/PBS supplemented with 25 mg Diazabicyclol (2,2,2)-octane (Aldrich-Chemie) (DABCO/glycerine/PBS; Alder and Schmid, 1987), and observed with a fluorescence microscope.

Electron microscopy

At each stage of the transformation of medusae into polyps, sets of 10 specimens were fixed overnight in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2) at 4°C, postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer before dehydration in an alcohol series, and embedded in Epon resin. Ultrathin sections were stained with 1% uranyl acetate and lead citrate for 15 min.

Isolation of tissues

To investigate the transformation potential of different medusa fragments, manubria and tentacular bulbs, as well as portions of radial and circular canals, tentacles, and portions of the interradial umbrella, were excised from *T. nutricula* medusae. In addition, the potential of exumbrellar epidermis, subumbrellar endoderm, and striated muscle was investigated as isolated tissue fragments or as a combination of these tissues (Fig. 1, A–I).

Tissue isolation was performed either mechanically with microscissors and watchmaker's forceps or by short incubation in collagenase (Schmid *et al.*, 1982). Isolation of striated muscle cells from *T. nutricula* proved to be harder than in *P. carnea,* probably due to stronger adhesion of the cells to the mesoglea (extracellular matrix, or

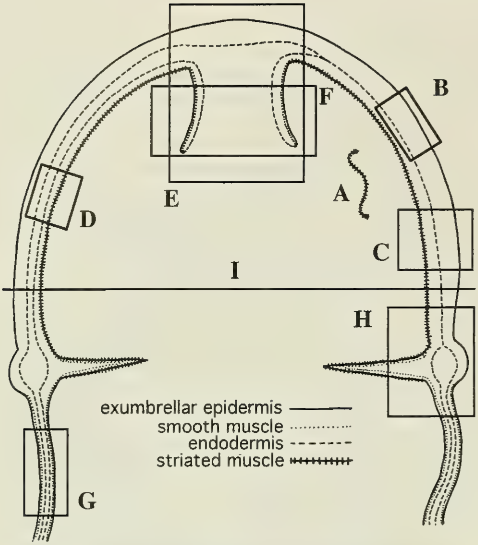


Figure 1. Scheme of dissection of tissues and fragments from *Turritopsis nutricula* medusae. A = fragments of isolated striated muscle tissue; B = exumbrellar epidermis + endoderm (interradial sectors); C = striated muscle + endoderm + epidermis (interradial sectors); D = striated muscle + endoderm of radial canals; E = manubrium + exumbrellar epidermis; F = manubrium only (middle-tip); G = tentacles; H = exumbrellar epidermis + velum + tentacular bulbs + tentacles; I = upper and lower halves of medusa.

ECM). The presence of occasional contaminating endodermal cells in the striated muscle isolates was tolerated because of their easy identification in both the light and the electron microscope (Schmid *et al.*, 1982). Tissue isolates or medusa fragments ($n = 20$) were cultured at 22°C in filtered (0.22 μm) seawater to which streptomycin (0.1 mg/ml), penicillin (100 U/ml), and chloramphenicol (0.01 mg/ml) were added. The culture media were changed daily. To induce transdifferentiation in some isolated portions of striated muscle, a post-isolation treatment with pronase (1.25 mg/ml seawater, Schmid, 1992) was also applied.

DNA replication

The pattern of DNA replication both in stable and in transforming medusae was determined to investigate whether differentiated cell types can resume cell cycle activity and be involved in the transformation process. Therefore, the rate and site of DNA replication in healthy and wounded medusae of both *T. nutricula* and *P. carnea* were investigated by pulse-labeling the animals or their fragments with 5-bromo-2-deoxyuridine (BrDU; Plickert and Krohier, 1988). For comparison, the same treatment was applied to entire *Bougainvillia muscus* medusae.

Animals were pooled in two groups. Group A: 60 medusae of *T. nutricula* and 60 medusae of *P. carnea* were cut in two with scissors (upper half of the bell with manubrium, lower half with ring canal and tentacles) and maintained at room temperature (20°C). Group B: 100 medusae of *T. nutricula,* 100 medusae of *P. carnea,* and 20 medusae of *B. muscus* were maintained as controls at room temperature.

At 0, 4, 8, 12, 24, 36, and 48 h, entire animals or medusa fragments were incubated for 4 h in BrdU medium (200 μmol/l in FSW) and then fixed in Lavdowsky's fixative (5 ml formaldehyde 37%, 2 ml glacial acetic acid, 25 ml ethanol 96%, 20 ml distilled water) for 1 h at room temperature. Then specimens were given two 10-min washes in PBS/Triton, incubated in freshly made 2 N HCl, and again washed twice, 10 min each time, in PBS/Triton. The BrdU label was detected immunocytochemically by incubation in (a) anti-BrdU antibody and (b) FITC-conjugated anti-mouse IgG antibody (Boehringer). Before observation with the fluorescence microscope, specimens were stained with Evan's blue in PBS/Triton for 2 min and with DAPI in PBS for 1 min before they were mounted in DABCO/glycerine/PBS.

A 4-h incubation in BrdU medium was applied to a single set of 20 medusae of *T. nutricula* in which the transformation process was initiated by an increase in water temperature (27°C).

The tissue distribution of labeled nuclei was recorded under a fluorescence microscope for each set of animals or fragments. To facilitate identification of the nuclei of striated muscle cells the fixed specimens were also stained with mAb 93, which is specific for the myosin heavy chain (Schuchert *et al.*, 1993).

In five specimens of both *P. carnea* and *T. nutricula* medusae, all DAPI-stained nuclei in the bell were counted.

Results

Anatomy of the medusa of Turritopsis nutricula

The bell height of newly liberated, 8-tentacled medusae of *Turritopsis nutricula* was about 1 mm. Sexual maturity was reached after 25–30 days at 20°C (or 18–22 days at 22°C), at a 16-tentacle stage with bell height of about 1.8–2 mm. Higher numbers of tentacles were never observed in our laboratory rearings, and literature on plankton records in the Mediterranean sea lacks reports of the "oceanic," larger form of *T. nutricula* medusae with a higher tentacle number (Brooks, 1886; Russell, 1953).

As in *Podocoryne carnea,* a layer of mononucleated striated muscle cells, uncontaminated by other cell types, lines the subumbrellar cavity of *T. nutricula* medusae (Fig. 2). This layer is in contact with a thin inner mesogloea. An extremely flattened, interradial subumbrellar plate

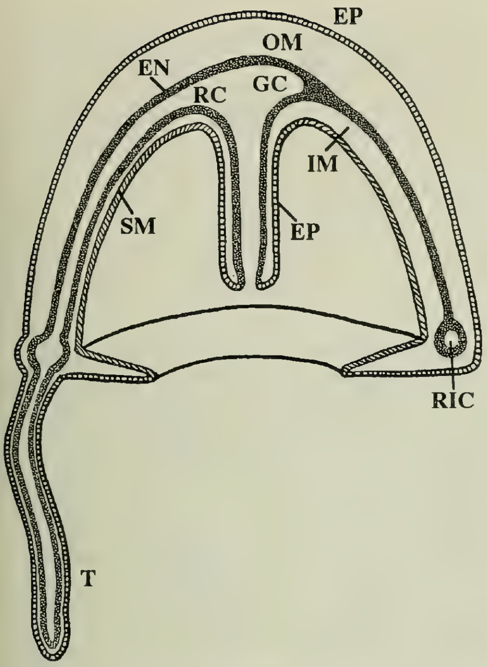


Figure 2. Anatomy of the medusa of *Turritopsis nutricula.* EP = epidermis, OM = outer mesogloea, EN = endoderm, IM = inner mesogloea, SM = striated muscle, GC = gastral cavity, RC = radial canals, RIC = ring canal, T = tentacle.

endoderm, connecting the endoderm of the radial canals (Delage and Hérouard, 1901; Bölsterli, 1977), divides the inner from the outer mesogloea (Fig. 2).

A first, dense nerve net of RFamide-positive cells is present in the epidermis of the manubrium lips. The net is connected through a few cells located above the radial canals to a second, larger network of nerve cells covering the circular canal, the tentacular bulbs and the tentacles. A second type of nerve cells was stained with the antibody against β1-integrin. These nerves form a ringlike structure that parallels the RFamide-positive nerve cells, but is restricted to the velar side of the circular canal and the tentacles. Smooth muscle cells were stained with mAb sm-1. The cells are present in the manubrium, the radial canals, the outer side of the velum, and the tentacles.

Transformation of medusae into stolons and polyps

Groups of 10 newly liberated medusae were separately reared and transformation was experimentally induced throughout development to sexual maturity either by (a) starvation, (b) sudden increase or reduction of water temperature (from 22°C to 17° or 27°C), (c) reduction of salinity (90% seawater, 10% distilled water, S = 33‰), or (d) mechanical damage of the bell with forceps or scissors. All immature medusae (up to a 12-tentacle stage) subjected to these different stresses regressed and transformed into stolons and polyps after expressing a cystlike stage. If reared under unstressed conditions, they never transformed spontaneously into cysts, stolons, or polyps. During gonad maturation (an irregular transition from 13- to 15-tentacle stages) the pattern of transformation was varied, and about 20%–40% of medusae skipped the cyst stage and transformed directly into stolons and polyps, no matter what the type of induction. Tentacle formation in this part of the cycle is rapid and changed even during the experiment. In contrast, all sexually mature medusae (at the 16-tentacle stage), even under the best culture conditions, regressed spontaneously and completely transformed into stolons and polyps.

The regression and transformation pathways of medusae into hydroid colonies are diagrammed in Figure 3, and they can be characterized as follows:

1. The developing medusa (Fig. 4) slowly enlarged its manubrium (Fig. 5), and the bell was either inverted (Fig. 6) or regressed without inversion, gradually contracting around the proximal base of the manubrium.
2. The tentacles and mesogloea degenerated and disappeared.
3. When transforming medusae were still immature, they regressed to a ball of tissue (a cyst) covered by perisarc (Fig. 7a). When cultured at 22°C, stolons were produced within 3 days (Fig. 7b). If temperature was progressively lowered to 14°C, the cysts rested for up to 3 months without losing their ability to produce stolons after the temperature was raised again to 22°C.
4. Mature medusae attached to the substrate by the apex (exumbrellar side) of the bell (Fig. 8). In general, spawning occurred shortly thereafter. A perisarc sheet and stolons soon appeared at the site of attachment.
5. Polyps were produced about 2 days after stolons had developed (Fig. 9a, b). The polyps were able to feed upon brine shrimp nauplii and to bud new medusae.

Transdifferentiation, regeneration, and transformation from isolated tissues and organs

To investigate whether the ability to transform to polyp structures is a property of the whole medusa or is restricted to only some of its organs and tissues, we dissected medusae into different body parts and cultured them (Fig. 1, A–I). Results of isolation experiments are summarized in Table I.

Medusa tissues and fragments unable to regenerate polyp tissues. Tissue fragments of mechanically isolated mononucleated cells of striated muscle (Fig. 1A; Table IA) occasionally transformed into flagellated cell types,

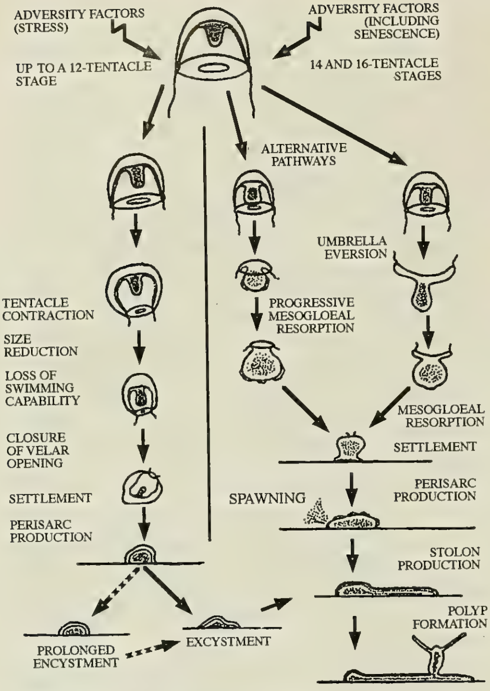

ADVERSITY FACTORS (STRESS)
ADVERSITY FACTORS (INCLUDING SENESCENCE)
UP TO A 12-TENTACLE STAGE
14 AND 16-TENTACLE STAGES
ALTERNATIVE PATHWAYS
UMBRELLA EVERSION
TENTACLE CONTRACTION
PROGRESSIVE MESOGLOEAL RESORPTION
SIZE REDUCTION
LOSS OF SWIMMING CAPABILITY
MESOGLOEAL RESORPTION
CLOSURE OF VELAR OPENING
SETTLEMENT
SETTLEMENT
SPAWNING
PERISARC PRODUCTION
PERISARC PRODUCTION
STOLON PRODUCTION
POLYP FORMATION
PROLONGED ENCYSTMENT
EXCYSTMENT

Figure 3. Pathways of transformation from medusa into polyp. Fate of stressed medusae up to 12-tentacle stage (left side), and alternative transformations of stressed or spawning medusae from a 14-tentacle or 16-tentacle stage (right side). The final product is always the polyp colony (bottom), directly or through a resting stage.

but polyp structures did not form. Myofibrils in these muscle cells progressively degenerated in the cytoplasm, while associated mitochondria clumped and degraded into lytic vesicles. However, mechanically isolated muscle cells (Fig. 1A) maintained their differentiated state (except in a few cases, see Table IA), even in the presence of radial canal endoderm (Fig. 1D, Table ID). On the other hand, the ECM-degrading pretreatment with collagenase and, overall, the pronase post-treatment induced a higher proportion of muscle cells to destabilize with degradation of striated myofibrils and transformation into flagellated cells (Fig. 10). As in *P. carnea* smooth muscle (Schmid and Alder, 1984), these transdifferentiating cells typically showed peripheral vacuoles filled with electron-dense material (Fig. 10).

When interradial fragments, consisting of exumbrellar epidermis and some endodermal cells (Fig. 1B; Table IB), were activated by collagenase treatment, flagella could form *de novo* (not shown). Occasionally, a thin perisarc layer was secreted (Fig. 11). However, stolon and polyp formation was never observed.

Interradial sections (Fig. 1C; Table IC) of the medusa bell containing striated muscle, endoderm of the subumbrella plate, exumbrellar epidermis, and both inner and outer mesogloea maintained a stable state for several days; cell dissociation then occurred, starting with the exumbrella cells, and the fragments fell apart.

Isolated manubria and tentacles (Fig. 1, F–G; Table I, F–G) did not transform into stolons and polyps. These fragments were highly stable for several weeks.

Medusan tissues and fragments regenerating polyp tissues. Full transformation into stolons and polyps was observed when excised manubria and tentacles additionally contained cells of exumbrellar epidermis and portions of the canal system (Fig. 1:E, H, I; Table I: E, H, I). In addition, bell fragments containing exumbrellar epidermis and portions of the radial or circular canal fully transformed into stolons and polyps (not included in Table I).

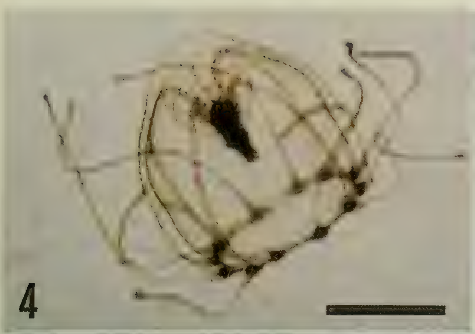
The pattern of DNA replication in different species of Anthomedusae

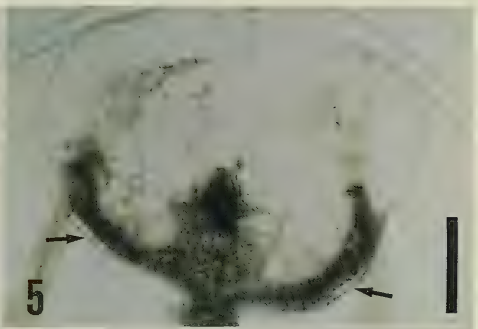
The location pattern of DNA-replicating nuclei in healthy, newly liberated or young (up to 10 days old) medusae of *T. nutricula* ($n = 100$), *P. carnea* ($n = 100$), and *B. muscus* ($n = 19$) was investigated by incubating animals of different ages every 4 h for 2 days in BrdU medium (see methods). No significant differences in the rate of DNA replication were observed in these 2 days.

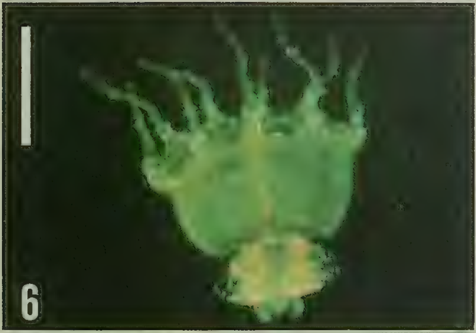
Medusae of all three species showed DNA replication in the manubrium, the tentacular bulbs and, to some extent, the proximal part of the tentacles. No specimen of *B. muscus* showed DNA replication in the umbrella proper (without circular canal and manubrium). Umbrellar DNA replication was detected in 28% of *P. carnea* specimens, but only 3% of the animals had more than 5 labeled nuclei, out of a total of 2300 ± 250 bell nuclei (Fig. 12). On the other hand, more than 75% of *T. nutricula* specimens showed DNA-replicating nuclei in the umbrella, 5% having from 50 to 250 labeled nuclei out of 5000 ± 500 bell nuclei (Fig. 13). Although labeled nuclei of exumbrellar epidermis cells were easily identified (Fig. 14), generally the subumbrellar endoderm could not be clearly distinguished from the striated muscle cells in untreated animals, so the DNA replication potential for these two cell types could not be accurately evaluated. However, striated muscle cells must be considered basically out of cell cycle activity (Schmid, 1992).

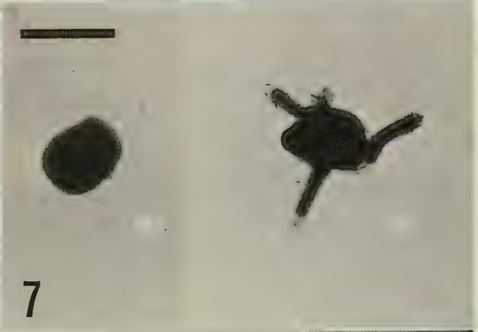
The effect of adversities on the rate of DNA replication

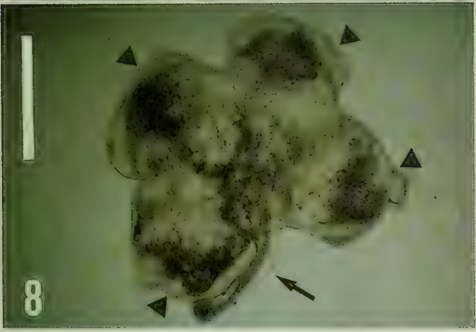
To investigate the influence of mechanical stress on DNA replication, medusae of both *P. carnea* and *T. nutricula* were cut in half (Fig. 1I) before BrdU incubation. No significant differences in the number of DNA-repli-











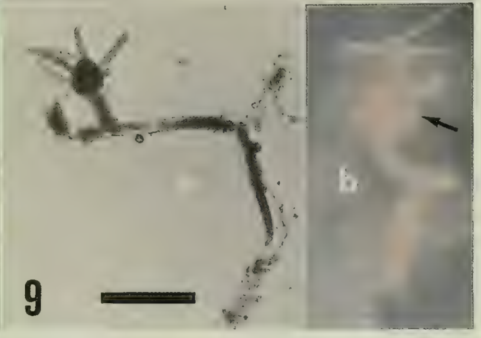


Figure 4. Medusa of *Turritopsis nutricula.* (12-tentacle stage). Scale bar: 1 mm.

Figure 5. Initiation of transformation: enlarged manubrium from a 16-tentacle medusa stage. Gonads in the process of maturation are already recognizable (arrows). Scale bar: 250 μm.

Figure 6. Everted medusa with extruded manubrium (bottom). Scale bar: 1 mm.

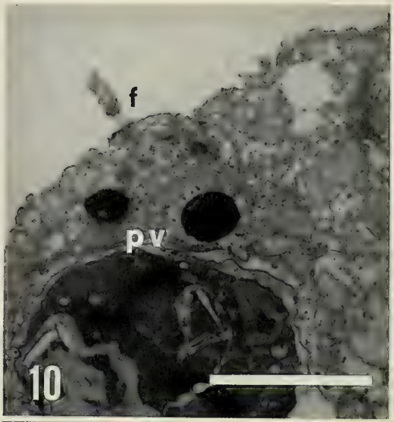
Figure 7. Encystment of an immature medusa of *T. nutricula:* (a) with outgrowing; stolons (b) 3 days after settlement of the medusa. Scale bar: 1 mm.

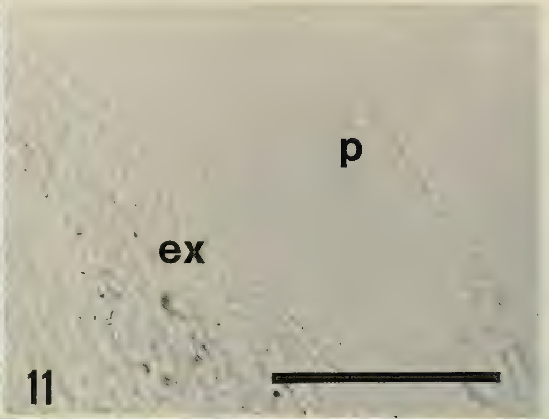
Figure 8. Male medusa of *T. nutricula* (reduced to a manubrial vestige settled on a glass slide) budding a stolon (arrow) while still retaining mature gamete clumps on four lobes (arrowheads). Scale bar: 1 mm.

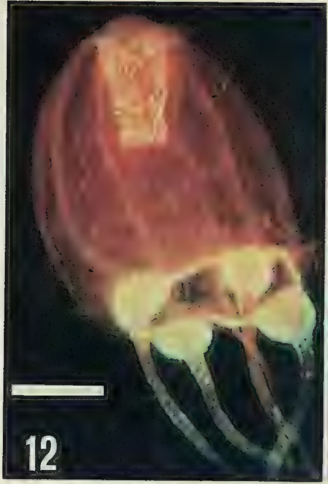
Figure 9. Polyps of *T. nutricula* on stolons developed from a transformed medusa. Preserved (a) and living specimens (b) with medusa cyst still recognizable (arrow). Scale bar: 1 mm.

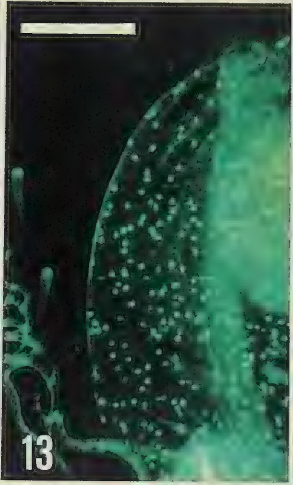
cating nuclei were detected between cut and control specimens in either species.

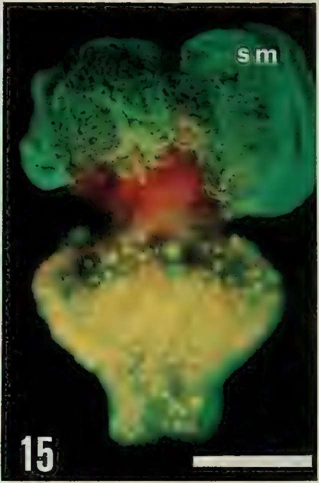
Before transformation of wounded medusae into polyp tissues, mechanical stress caused umbrellar eversion of upper half-cut parts of *T. nutricula,* so that the striated muscle cells were stretched outwards (Fig. 15),

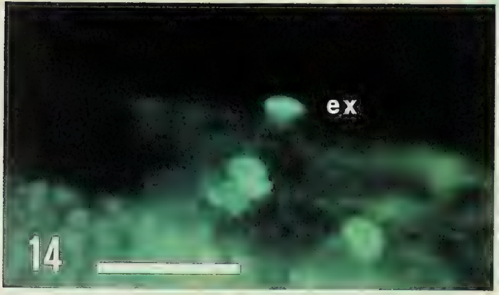












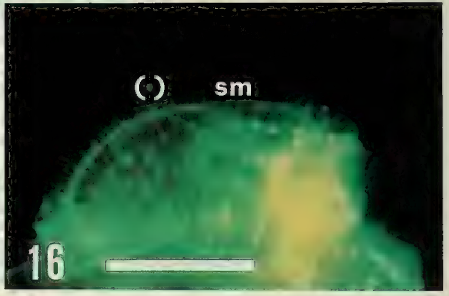


Figure 10. Electron micrograph of an isolated fragment of activated striated muscle tissue transforming into smooth-muscle-like, flagellated cells. pv = peripheral vesicles, f = flagellum. Scale bar: 5 μm.

Figure 11. Destabilized exumbrellar epidermis (ex) from transforming *Turritopsis nutricula* medusa, showing large perisarc secretion (p). Scale bar: 80 μm.

Figure 12. *Podocoryne carnea* medusa. BrdU staining of DNA replicating nuclei (courtesy of G. Plickert). Replicating nuclei are concentrated in the manubrium and tentacular bulbs. Scale bar: 500 μm.

Table I

Destabilization of and stolon formation from isolated medusa fragments (A–I, see Fig. 1)

Isolate composition	Isolation/treatment procedure	Destabilization	Stolon formation
A Striated muscle	Mechanical	+	–
	Collagenase	++	–
	Pronase*	+++	–
B Exumbrellar epidermis + endoderm	Collagenase	+	–
C Striated muscle + endoderm + epidermis (interradial sectors)	Mechanical	–	–
D Striated muscle + radial canals	Mechanical	–	–
E Manubrium + exumbrellar epidermis	Mechanical	+++	+++
F Manubrium only (middle–tip)	Mechanical	–	–
G Tentacles	Mechanical	–	–
H Ex. epidermis + tent. bulbs + tentacles	Mechanical	+++	+++
I Upper half of medusa	Mechanical	+++	+++
Lower half of medusa	Mechanical	+++	+++

Striated muscle was isolated either mechanically or with collagenase. Occasionally, mechanically isolated fragments were treated with pronase (*). Number of isolates: A–H = 20; I = 60. Destabilization was scored either by formation of flagella or by production of perisarc, adhesion of fragments to the bottom of culture plates, or both. Percentage of destabilized fragments: + = <5%; ++ = 5%–50%; +++ = 50%–100%.

being more distinguishable from the subumbrellar plate endoderm. In this case, DNA replication was easily detected in a few nuclei belonging to the striated muscle sheet (Fig. 16).

Also, DNA replication was unequivocally resumed in some nuclei of well-differentiated muscle cells (Fig. 17a, b) from unwounded medusae, whose transformation was induced by heat stress.

Discussion

Does death occur in Turritopsis nutricula?

Turritopsis nutricula is a unique medusa and its transformation potential is unparalleled within the vast array of life-cycle patterns found in cnidarians. This is the first known case of a metazoan being capable of reverting completely to a clonal life stage after having achieved sexual maturity in a solitary stage.

Because all *T. nutricula* medusae regularly underwent transformation, we must assume that organismic death does not occur in this species. Hydrozoans are modular organisms in their polyp stage, but the medusa stage is unitary. Modular organisms have a potentially indefinite life span, but this is usually impossible for nonmodular ones, in which the onset of sexual reproduction ultimately leads to death (reviewed in Stearns, 1992). In planarians, aging is retarded through reduction in size, or degrowth, the regressing adults resembling juveniles in both morphology and physiology (reviewed in Calow, 1978). In bryozoans, colonies rejuvenate by dismantling their bodies and rebuilding them from undifferentiated cells (Gordon, 1977). Colony size is generally reduced in social and compound ascidians by the resorption of zooids, leaving potential buds of presumptive germinative tissue that quickly develop into new blastozooids under favorable conditions ("survival budding" as overwintering tactic: Nakauchi, 1982). All these processes, however, imply only a reorganization of the original morph. Instead, the transformation of *T. nutricula* may be considered a metamorphosis, though in a direction opposite to the usual ontogenetic path. In this same sense, a case of reversible metamorphosis was previously reported in corals in which primary polyps revert to "secondary" planula larvae when suitable conditions for

Figure 13. *T. nutricula* medusa, portion of bell shown. BrdU staining of DNA replicating nuclei (incubation: 30 min). Besides manubrium (arrow) and bulbs (arrowheads), labeled nuclei are widespread throughout the whole umbrella. Scale bar: 500 μm.

Figure 14. DNA-replicating cell of exumbrellar epidermis (ex) from the margin of *T. nutricula* medusa bell. Scale bar: 50 μm.

Figure 15. Half-cut, upper part of BrdU-incubated medusa of *T. nutricula* with extruded manubrium (bottom) and outwardly strained striated muscle sheet (sm). Striated myofibrils stained with mAb 93. Scale bar: 40 μm.

Figure 16. Outwardly strained striated muscle sheet (sm) from half-cut, upper part of BrdU-incubated medusa of *T. nutricula*. Brackets point out a DNA-replicating nucleus from the striated muscle sheet. Scale bar: 200 μm.

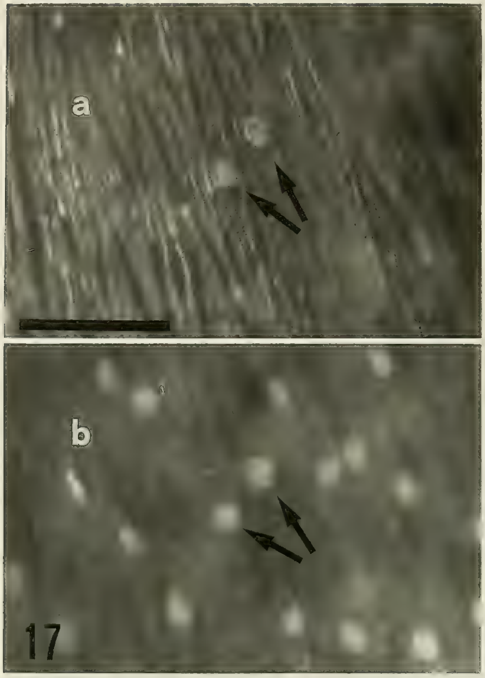


Figure 17. Striated muscle cells from BrdU-incubated, whole medusa of *Turritopsis nutricula* undergoing transformation into polyps after rearing at T = 27°C. (a–b) same frame: (a) two DNA-replicating, BrdU-labeled nuclei (arrows), with mAb 93-stained myofibrils running around them; (b) DAPI staining showing the two BrdU-labeled cells (arrows) surrounded by nonreplicating, striated muscle cells. Scale bar: 50 μm.

polyp colony growth are not encountered after "primary" planulae settlement (Richmond, 1985). However, that ontogenetic reversal occurs at the beginning of development, before colony growth and sexual reproduction, and does not involve a return to clonal or larval stages from aclonal ones. Similarly, scyphozoan ephyrae are reportedly able to transform into polyps under starvation conditions (Hadzi, 1912). As already mentioned, this "metamorphic" potential is present in the first stages of medusan development of other hydroidomedusan species, but is lost before the medusa detaches from the polyp (Müller, 1913; Hauenschild, 1956; Frey, 1968; Kakinuma, 1969; Schmid, 1972). In contrast, in *T. nutricula* each stage of medusa development can shift to hydroid structures as a response to adverse conditions, including senescence. This is an exceptional situation, like that of a hypothetical insect imago able to reverse to a larval stage after sexual reproduction.

Stem cells (interstitial cells) and transdifferentiation

The change of medusae into polyps requires differentiation of new cell types and major reorganization of tissues. New cell types could arise either through a differentiation of uncommitted stem cells of the medusa or by cellular trandifferentiation of differentiated medusa cells.

The presence of a population of continuously proliferating cells (stem cells, interstitial or I-cells) was long thought to play a key role in asexual reproduction, in regenerative processes, and in the apparent lack of senescence observed in both colonial and solitary cnidarians (Brien, 1953; Crowell, 1953; Comfort, 1956; Brock and Strehler, 1963; Toth, 1969; Hughes, 1987; Buss, 1987). These cells are characterized by their histological features, but their state of commitment cannot be determined by isolation and cloning experiments. The role of interstitial cells is best understood in *Hydra* spp. in which these cells are able to differentiate into nematocytes, nerve cells, and germ cells (Tardent, 1978; Werner, 1984). It seems that, as a gross morphological category, interstitial cells include partially differentiated stem cells (Bode, 1992), but the question of whether their repertoire includes secretory cells is still unresolved (Smid, 1984). In any case, their influence on morphogenetic processes seems to be rather limited. Interstitial-cell-free *Hydra* polyps are able to regenerate fully and reproduce asexually (Brien, 1955; Diehl and Bouillon, 1966; Marcum and Campbell, 1978).

Recruitment of differentiated cells for transdifferentiation and regeneration processes was demonstrated in the medusa *Podocoryne carnea* (for review see Schmid, 1992). In that case, isolated and activated endoderm or striated muscle cells can resume DNA replication, transdifferentiate to several new cell types, and regenerate *in vitro* medusa organs. However, they are unable to regenerate polyp organs such as stolons or perisarc-secreting tissues (Schmid, 1992). Transdifferentiation in hydroidomedusae might be a common phenomenon in budding and regeneration, especially where no or few I-cells are located (Brien, 1941). Some hydroidomedusae belonging to quite different families (*e.g., Zanclea prolifera, Teissiera australis, Bougainvillia vervoorti, Proboscidactyla ornata, Cytaeis* spp., *Eirene elliceana, Eucheilota paradoxica, Clytia mccradyi*) have the ability to bud (but not to metamorphose into) polyp structures before sexual reproduction. Also, direct medusa budding from liberated medusae is known in several species at three alternative sites: (1) manubrial wall (*e.g., Sarsia gemmifera, Eucodonium brownei, Podocoryne minima, Bougainvillia niobe, Rathkea octopunctata, Lizzia blondina*); (2) radial canals (*e.g., Proboscidactyla ornata, Eucheilota paradoxica*); and (3) tentacle bulbs (*e.g., Niobia dendrotentaculata, Hybocodon prolifer, Sarsia prolifera*). These observations led to the assumption that both epidermis and endoderm are basically totipotent (Berrill, 1950). Indeed, manubrial budding of medusae in *Rathkea octopunctata* seems to derive from the sole contribution of differentiated epidermal cells, with no aid from the I-cell compartment (Bouillon, 1961–1962). However, in all the other cases, the contribution of transdifferentiation processes is still unstudied.

Transformation of T. nutricula *medusae*

The excision experiments and the DNA-replication pattern in the nontransforming and the transforming medusa indicate that the transformation process does not depend exclusively on stem cells. In fact, isolated manubria, containing a large population of replicating stem cells (Tardent, 1978), are unable to tranform into stolons and polyps. Transformation occurs only in fragments that contain tissue of the exumbrella and of the canal system (the radial canals or the ring canal). Interstitial cells do not occur in the exumbrellar epidermis and are not prominent in the canals. The exumbrellar epidermis is the only tissue able to transform into the perisarc-secreting, epidermal tissue of the stolons; thus, we must conclude that the exumbrellar epidermal cell type clearly undergoes transdifferentiation. However, its potential is limited because the fragments are not able to reconstitute the endoderm needed to complete the transformation process. The endodermal lining of the canals evidently harbors the cells that give rise to the endoderm of stolons and polyps.

Therefore, the main question is what cell type (or types) in the transforming medusa or fragment is able to generate the missing cell types (sensory cells, myoepithelial cells, cnidocytes) needed to complete the transformation process. The presence of a few scattered interstitial cells cannot be excluded, and these cells could be responsible for the completion of the cell-type inventory. However, the available information is insufficient to allow discussion of this possibility. On the other hand, transdifferentiation from one or several differentiated cell types (secretory or digestive cells) could be an additional alternative. Transdifferentiation in general requires DNA replication (for review see Okada, 1991). Compared to the bell of *B. muscus,* where no DNA replication was shown, and of *P. carnea,* where DNA replication was scarce, in *T. nutricula* the cells of the bell can still undergo intensive DNA replication. This is especially true for the cells of the exumbrella, the endoderm of the radial canals, and those of the subumbrellar plate endoderm. Traumatization and heating experiments in *T. nutricula* showed DNA replication to occur in striated muscle nuclei too, suggesting that even some of these cells can contribute by transdifferentiation to the transformation process.

Conclusions

According to our results, the transformation of mature medusae of *T. nutricula* into stolons and polyps is an established trait in the life cycle of this species. It is not known whether these processes occur under natural conditions, because they take place in a rather short time and are difficult to observe in the field. Laboratory observations, however, demonstrate that *T. nutricula* has a transformation potential that has never been recorded in any other cultured species, and it seems improbable that such potential is expressed only under laboratory conditions. Excision experiments demonstrate that cells of the exumbrella and of the gastrovascular system are required for transformation. The perisarc-secreting cells of the stolons appear to be formed by transdifferentiation of some cells of the exumbrella. A contribution by differentiating stem cells (interstitial cells) cannot yet be ruled out. Comparative studies on the pattern of replicating nuclei among different species demonstrate that the cell types of the bell, including well-differentiated striated muscle cells, in *T. nutricula* have retained the ability to replicate. Further studies are required to investigate the role of DNA replication in the transformation process and the mechanisms that regulate this life-cycle reversal at the molecular level.

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Homeotic Transformation of Crab Walking Leg into Claw by Autotransplantation of Claw Tissue

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Abstract. Homeotic transformation is defined as transformation of one body part into the likeness of something else. By autotransplantation of crab claw tissue into the autotomized stump of the fourth walking leg, the stump can regenerate a complete claw. Frozen claw tissue, sham operation, or walking leg tissue had no such activity. Contralateral autotransplantation of claw tissue into the autotomized stump of the fourth walking leg can induce the regeneration of a claw with normal handedness. Most of the transformed claws combined features of the claw and the walking leg, suggesting that both host and donor tissues play a role in regeneration. Three possible mechanisms that might account for limb transformation are discussed. Simple intercalary regeneration does not explain all of the observations, but some regulatory events might be taking place during regeneration. Two other processes—secretion of some morphogen by the claw tissue and alteration in the expression of *Hox* genes—offer alternatives that might explain the results of this study.

Introduction

Limbs are important structures for the study of pattern formation. Intensive studies have been conducted on pattern formation of the anteroposterior axis of the limb (reviewed by Tickle, 1994; Tabin, 1995). Little is known, however, about how different limbs, such as the forelimb and hindlimb, are generated. Crabs have easily distinguishable forelimbs, the claws, and hindlimbs, the walking legs. In addition, they have the ability to regenerate missing limbs, which provides a favorable model for the study of pattern formation. A crab has five pairs of thoracic limbs: the first pair terminates in pincerlike structures called claws or chelipeds; the second to fifth thoracic limbs lack the pincerlike structures and are called walking legs. The major difference between a claw and a walking leg is the presence of the pollex in a claw. The pollex is an extension of the manus, which is the penultimate segment of the crab limb (Fig. 1).

The crab has the ability to autotomize an injured limb and regenerate it (reviewed by Hopkins, 1988). Limb autotomy results from the fracture of a preformed breakage plane within the second limb segment, the basiischium (Findlay and McVean, 1977). Distal to the autotomized limb is a membranous connective tissue septum that immediately closes the gap created by autotomy. This septum covers the autotomized stump, which is composed of the coxa and basis segments. Epithelial and hemolymph cells migrate to the wound to further enclose it. A few days after autotomy, the epidermal and migrant cells under the scab begin to proliferate and a regenerating blastema emerges (Emmel, 1910; Adiyodi, 1972). The blastema continues to grow and the cells at the apex and future segments of the blastema begin to invaginate. The invagination process of the epithelial cells results in formation of the dactyl, pollex, and other segments of the claw (Emmel, 1910).

The undifferentiated blastema of the crab has its shape specified already. Following transplantation of the claw blastema between female and male or between two species of fiddler crabs, the shape of the regenerated claw resembled that of the donor (Trinkaus-Randall and Mittenthal, 1978; Trinkaus-Randall, 1982). The morphology of the regenerated limbs can be influenced by the donor tissue. In crayfish, the first three pairs of thoracic limbs are chelated. When the basis segment of the first claw is transplanted into the autotomized stump of the second claw or *vice versa,* the regenerated limb may have the morphology of the limb of the donor, the host, or both (Mittenthal, 1980). The crayfish has the ability to intercalate the missing part of the limb. When the dactyl segment

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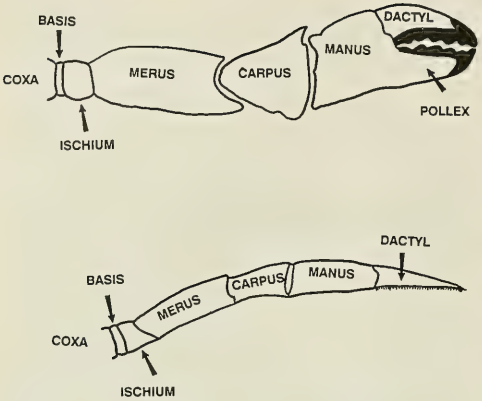

Figure 1. Structure of a claw (top) and a walking leg (bottom).

was inserted into the proximal part of manus segment, jointed and jointless regenerates formed (Mittenthal, 1981).

Because the stump of the claw always regenerates into a claw and the morphology of the regenerated limb can be affected by the grafting tissue, we propose that there are some components in the claw tissue—cells or morphogen secreted by the cells—that are responsible for claw patterning during limb regeneration. If the cells or composition of the cells in the stump of a walking leg are changed, the identity of the walking leg might be changed after limb regeneration. This hypothesis can be tested simply by autotransplantation of claw cells to the stump of the walking leg. In this study, we are interested in the following questions: Can the walking leg be transformed into a claw by autotransplantation of claw tissue? Which segments of the claw have the claw-transforming activity? Does claw tissue contain sufficient morphogen for claw patterning? Is regeneration an intercalary process?

Materials and Methods

Animals and dissection

The following *Cancer* spp. were used: *C. anthonyi* (range of carapace width = 14.1–52.5 mm, mean ± SD = 31.8 ± 6.7 mm; *n* = 39), *C. gracilis* (range = 9.4–16.0 mm, mean ± SD = 13.2 ± 0.4 mm; *n* = 347), and *C. productus* (range = 13.3–57.8 mm, mean ± SD = 29.8 ± 4.3 mm; *n* = 106) Specimens were collected subtidally with a hand net in Bodega Bay Harbor, California. The crabs were kept in individual compartments to prevent cannibalism (Conklin and Chang, 1993) and were fed brine shrimp, shrimp meat, or fish every other day. Dissecting tools, scissors, forceps, and insect pins were sterilized with 70% ethanol. Animals were forced to autotomize either three limbs (one claw and both fourth walking legs), or five limbs (one claw and both third and fourth walking legs). Autotomy was achieved by crushing the manus or carpus segment of the limb with forceps. For donor tissues, dactyl, pollex, and ischium segments of the claw were cut off with scissors and put into a concave slide with crab saline (440 m*M* NaCl, 11.3 m*M* KCl, 13.3 m*M* $CaCl_2$, 26 m*M* $MgCl_2$, 23 m*M* Na_2SO_4, 10 m*M* HEPES, pH 7.4 with NaOH, 100 units/ml penicillin, 0.1 mg/ml streptomycin; Cooke *et al.*, 1989). Tissues from each segment were dissected out.

Tissue autotransplantation in C. gracilis

Two kinds of tissue transplantation were performed. For lateral autotransplantation, claw tissues were autotransplanted into the stump of an ipsilateral walking leg; *e.g.*, left claw tissue was autotransplanted into the coxa and basis segments of the left walking leg of the same animal. For contralateral autotransplantation, claw tissues were autotransplanted into the stump of the contralateral side of the walking leg; *e.g.*, left claw tissue was transplanted into the stump of the right walking leg of the same animal. Autotransplantation was performed by placing the tissue under the autotomy septum of the fourth walking leg stump with a pair of fine forceps under a dissecting microscope. An insect pin with a blunt end was used to push the claw tissue into the stump. After manipulation, animals were returned to seawater.

To determine which claw segments have the activity to induce the stump of a walking leg to regenerate a claw, various claw tissues (dactyl, pollex, or ischium) were autotransplanted into the stump of the fourth walking leg. To test whether dead tissue contains enough morphogen for inducing the claw pattern, claws were frozen at −70°C for one day and thawed at room temperature. Pollex tissues of the frozen claws were dissected out and autotransplanted into the stump of the fourth walking legs. To see whether the regeneration process was an intercalary one, claw dactyl or pollex tissue was contralaterally autotransplanted into the stump of the fourth walking leg.

Three controls were performed. First, unmanipulated controls were stumps of the fourth walking leg that did not receive any tissue. Second, sham operations were performed in which the stumps of the fourth contralateral walking leg of the experimental animals received three insertions from a pair of forceps. Third, stumps of the fourth walking leg received implants of their own dactyl tissue.

Multiple tissue autotransplantation in C. productus

This experiment had three purposes: first, to confirm the results with *C. gracilis;* second, to verify the phenomenon that the walking leg can be transformed into a claw in another species of crab; and third, to test the hypothesis that the percentage of claw transformation is proportional to the amount of claw tissue transplanted. In this experiment, either (1) different portions of the claw tissue (dactyl, pollex, or ischium) or (2) combinations of various claw tissues (dactyl + pollex; dactyl + pollex + ischium) were autotransplanted into the stump of the fourth walking leg of *C. productus*. The contralateral sham operation, autotransplantation of frozen claw tissues (dactyl + pollex), and unmanipulated controls were also performed. In addition, walking leg tissues (dactyl + ischium + joint between the merus and carpus) were contralaterally autotransplanted into the stump of the fourth walking leg.

Multiple limb autotransplantation in C. anthonyi

Animals were forced to autotomize five limbs—one claw and the third and fourth walking legs on both sides. To test the possibility that both the third and fourth walking leg can be transformed into a claw, the stumps of the right or left third and fourth walking legs received the dactyl or pollex tissues of the claw. Contralateral autotransplantations, contralateral sham operations, and unmanipulated autotomized controls were also conducted.

Results

Tissue autotransplantation in C. gracilis

Of 299 experimental animals, including those that received frozen tissue autotransplantation, 195 crabs (65.2%) died before the limb regenerated (Table I). Most of those deaths were due to postoperative trauma. Among the surviving experimental crabs, six regenerated claws (Fig. 2A–C) from the stump of the fourth walking leg (hereafter called the transformed claw). Tissues from the dactyl, pollex, and ischium tissues of the claw all showed claw-transforming activity (the activity to transform the walking leg into the claw). Contralateral autotransplantation of dactyl or pollex tissue of the claw gave rise to either a claw (Fig. 2C), a normal walking leg, or a branched walking leg (Fig. 2D). This branched walking leg was induced by the contralateral dactyl tissue of the claw. The extra limb structure of the branched limb had the appearance of a truncated claw that was grown from the merus segment of the walking leg.

Among the transformed claws, one was a complete claw (Fig. 2A, B) that showed a high degree of similarity to the authentic claw. Not only did its dactyl and pollex bear teeth, but its subsegments were also larger than the walking leg and it was capable of bending like an authentic claw. The walking leg bends in the dorsal-ventral plane and the claw bends in the lateral plane. Five of six transformed claws were chimeras of a walking leg and claw (Fig. 2C). They showed only partial similarity to an authentic claw. In three of the chimeric transformed claws, both the dactyl and pollex of the transformed claw had teeth, but its subsegments were slender and jointed like the walking leg. Alternatively, in two of the chimeric transformed claws, only the pollex and not the dactyl had teeth (Fig. 2C).

Table I

Summary of tissue autotransplantation experiment in Cancer gracilis *(mean carapace width = 13.2 ± 0.4 mm)*

				Types of limb regenerated*		
Operation†	*n*	Death	Survival	Normal	Abnormal	Claw
D → W4	46	34	12	11	0	1
Dc → W4	48	34	14	11	2	1
P → W4	41	29	12	9	3	0
Pc → W4	39	29	10	7	0	3
Pf → W4	50	25	25	25	0	0
I → W4	38	27	11	10	0	1
W4 → W4	37	17	20	19	1	0
Sham	299	195	104	104	0	0
Control	48	7	41	41	0	0

* Normal: normal walking leg; abnormal; abnormal walking leg; claw: a claw or clawlike structure was regenerated from the stump of the fourth walking leg.

† All operations were performed on the fourth walking leg (W4).

D → W4: Dactyl tissue of the claw was autotransplanted into the stump (coxa and basis segments) of W4.

Dc → W4: Dactyl tissue from the contralateral claw was autotransplanted into the stump of W4.

P → W4: Pollex tissue of the claw was autotransplanted into the stump of W4.

Pc → W4: Pollex tissue from the contralateral claw was autotransplanted into the stump of W4.

Pf → W4: Frozen pollex tissue was autotransplanted into the stump of W4.

I → W4: Ischium tissue of the claw was autotransplanted into the stump of W4.

W4 → W4: Dactyl tissue of the fourth walking leg was autotransplanted into the stump of W4.

Sham: Contralateral sham operation; the stump of the contralateral W4 received three insertions from a pair of forceps.

Control: Stump of W4 did not receive any tissue.

Abnormal walking legs were regenerated in two animals that received pollex tissue. They regenerated Y-shaped and fork-shaped walking legs (Fig. 2E, F). Autotransplantation of dactyl tissue from the fourth walking leg gave rise to an abnormal walking leg in one instance. This abnormal limb had an enlarged propodus segment. Control and sham-operated animals all regenerated normal walking legs. Among the six crabs with transformed claws (Table I), four claws were autotomized at the first or second molts. After autotomy, they all regenerated normal walking legs from the stump of the fourth walking leg. The remainder of the crabs with transformed claws still maintained the claw structure after four post-transformation molts.

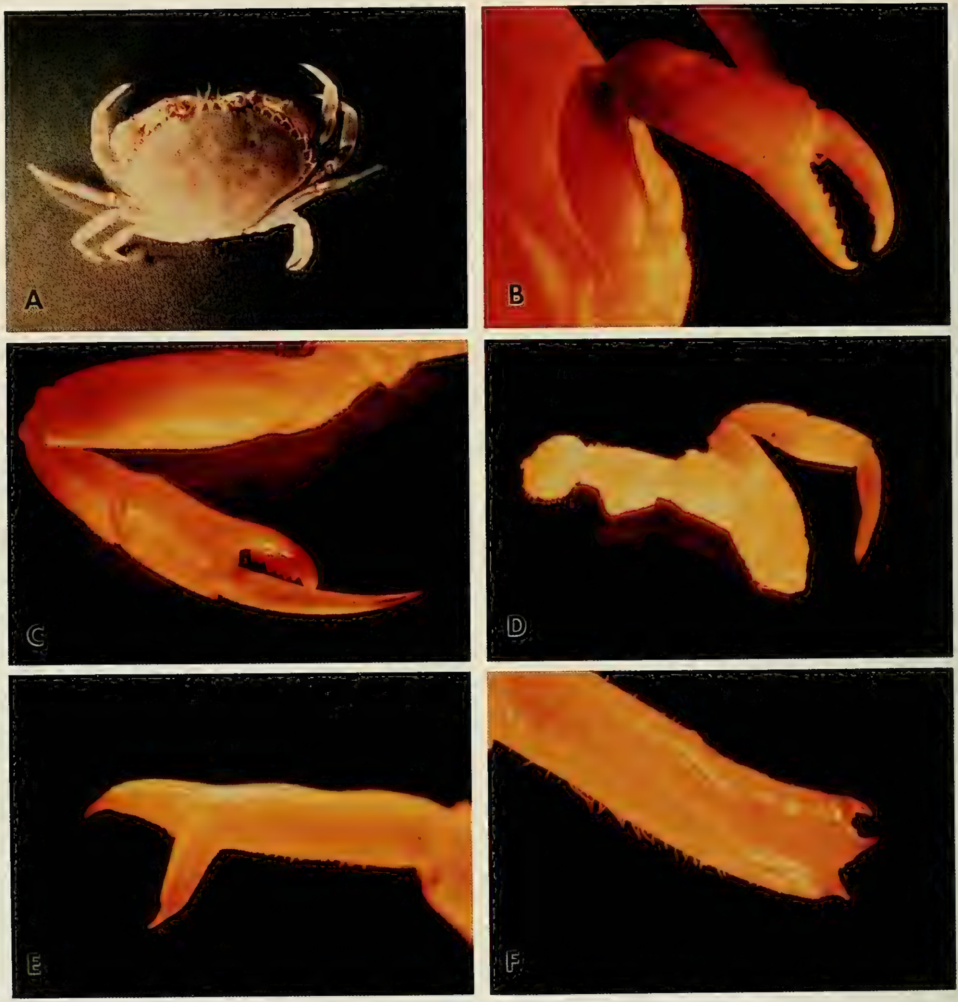

Figure 2. Regenerated limbs of the fourth walking leg in *Cancer gracilis* after receiving the autotransplant of claw tissue. (A, B) The stump of the fourth right walking leg regenerated a completed claw. (C) Chimeric claw. (D) Branched walking leg. (E) Y-shaped walking leg. (F) Fork-shaped walking leg.

Multiple tissue autotransplantation in C. productus

Of 98 experimental animals, 76 (77.6%) survived (Table II). Six of those crabs regenerated a claw (Fig. 3A) from the stump of the fourth walking leg. Of the transformed claws, one was a complete claw (Fig. 3A) and five were chimeras of the walking leg and claw. Twenty animals that received live claw tissue regenerated abnormal walking legs (Fig. 3B–E). Those abnormal walking legs included branched (Fig. 3B), bent (Fig. 3C), circular (Fig. 3D), and partial clawlike structures (Fig. 3E). The branched walking leg (Fig. 3B) was induced by lateral autotransplantation of pollex tissue of the claw. The bent walking leg (Fig. 3C) was induced by lateral autotransplantation of the dactyl tissue of the claw. The circular

Table II

Summary of multiple tissue autotransplantation experiment in Cancer productus *(mean carapace width = 29.8 ± 4.3 mm)*

				Types of limb regenerated*		
Operation[†]	*n*	Death	Survival	Normal	Abnormal	Claw
D → W4	16	5	11	5	6	0
P → W4	14	2	12	9	2	1
I → W4	14	4	10	10	0	0
D + P → W4	16	5	11	4	5	2
D + P + I → W4	16	3	13	3	7	3
F (P + D) → W4	15	3	12	12	0	0
W4c → W4	7	0	7	5	2	0
Sham	91	22	69	69	0	0
Control	8	0	8	8	0	0

* Normal: normal walking leg; abnormal: abnormal walking leg; claw: a claw or clawlike structure was regenerated from the stump of the fourth walking leg.

[†] All operations were performed on the fourth walking leg (W4).

D → W4: Dactyl tissue of the claw was autotransplanted into the stump of W4.

P → W4: Pollex tissue of the claw was autotransplanted into the stump of W4.

I → W4: Ischium tissue of the claw was autotransplanted into the stump of W4.

D + P → W4: Both dactyl and pollex tissues of the claw were autotransplanted into the stump of W4.

D + P + I → W4: Tissues of dactyl, pollex, and ischium of the claw were autotransplanted into the stump W4.

F (D + P) → W4: Frozen tissues of both dactyl and pollex of the claw were autotransplanted into the stump of W4.

W4c → W4: Tissues of dactyl, ischium, and joint between the merus and carpus of the fourth contralateral walking leg were autotransplanted into the stump of W4.

Sham: Contralateral sham operation; the stump of the contralateral W4 received three insertions from a pair of forceps.

Control: The stump of W4 did not receive any tissue.

walking leg (Fig. 3D) was induced by lateral autotransplantation of the pollex and dactyl tissues. The clawlike walking leg (Fig. 3E) had an enlarged merus segment that was induced by lateral autotransplantation of pollex, dactyl, and ischium tissues. Combinations of abnormalities, such as a limb that was both bent and branched, were occasionally observed. However, there was no consistent pattern in the generation of these abnormalities.

Thirty-one animals that received live claw tissues regenerated normal walking legs (Table II). Again, autotransplantation of frozen dactyl plus pollex tissues of the claw did not induce the stump of the fourth walking leg to regenerate a claw. Both control and contralateral sham-operated animals regenerated normal walking legs. Autotransplantation of single claw tissues, either dactyl, pollex, or ischium, gave rise to one claw (3.0% of the survivors). Autotransplantation of multiple tissues, (dactyl + pollex) or (dactyl + pollex + ischium), gave rise to five claws (20.8% of the survivors). There was a significant difference in claw transformation between single and multiple claw-tissue autotransplantations. Autotransplantation of multiple tissues of the fourth contralateral walking leg to the stump of the fourth walking leg regenerated either a normal walking leg, one that was bent, or a walking leg with two dactyls (Fig. 3F). Those two dactyls were jointed at an enlarged manus segment. The rest of the subsegments of the walking leg were normal.

Multiple limb autotransplantation in C. anthonyi

Of 32 experimental animals, 23 survived (71.9%). Among those survivors (23 crabs with 46 transplanted stumps), 34 of 46 stumps (73.9%) regenerated normal walking legs, six stumps (13%) regenerated abnormal legs, and six stumps (13%) regenerated claws (Fig 4A). Those transformed claws were all chimeric and regenerated in only four crabs. Two crabs had regenerated claws from the stumps of both the third and fourth walking legs. One crab had a regenerated claw from the stump of the third walking leg, and one crab had a regenerated claw from the stump of the fourth walking leg. Among the crabs with abnormal legs, one leg had two pollexes and three dactyls (Fig. 4B), one had a Y-shaped dactyl (Fig. 4C), two had clawlike dactyls (Fig. 4D), and the rest of the abnormal legs had a curved structure. The morphology of the Y-shaped dactyl was in-between that of the Y-shaped and fork-shaped regenerated limbs in *C. gracilis.* Unmanipulated controls (seven survivors, one death) and contralateral sham-operated crabs (46 stumps) all regenerated normal walking legs.

Discussion

Although the percentage of claw transformation that we observed was low (11.1% of the survivors of the live claw tissue implants), we have, however, succeeded in transformation of a walking leg into a claw in three species of *Cancer* crabs. The results confirmed that the claw tissue has some components that are responsible for claw patterning during regeneration. A complete claw, a chimeric claw, a walking leg with a clawlike dactyl, a Y-shaped walking leg, and a fork-shaped walking leg might represent different degrees of claw transformation. Although naturally occurring abnormalities have been found in decapod claws, no examples of limb transformation were observed (Shelton *et al.,* 1981). Furthermore, sham-operated crabs all regenerated normal walking legs. This suggests that limb transformation of crabs is not due to the traumatic effects of transplantation. Dactyl, pollex, and ischium tissues of the claw all have claw-transforming activity, which suggests that tissues of other claw segments might also have similar activity. Claw ischium tissue had the lowest activity compared to tissue from the pollex or dactyl. The relatively

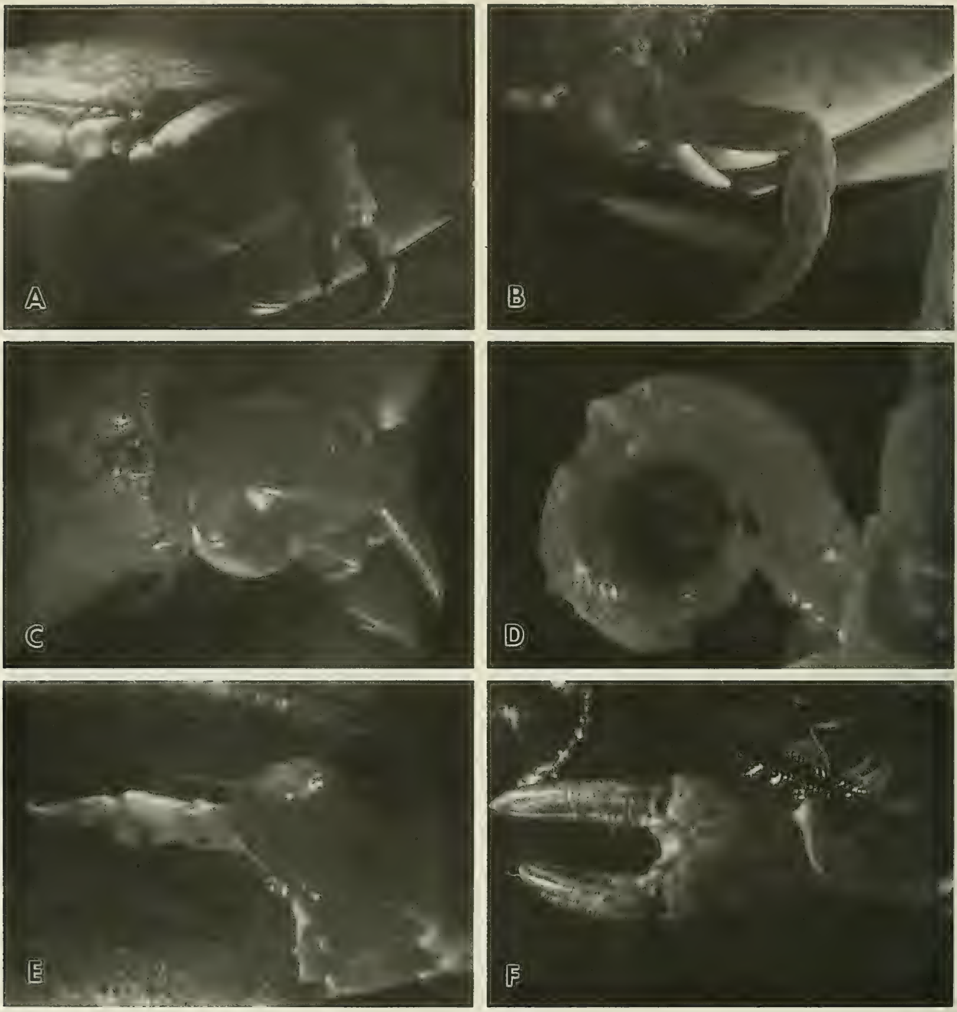

Figure 3. Regenerated limbs of the fourth walking leg in *Cancer productus.* (A) Complete claw regenerated from the stump of the fourth right walking leg. (B) Branched walking leg. (C) Bent walking leg. (D) Circular walking leg. (E) Regenerated walking leg with a clawlike merus segment. (F) Regenerated walking leg with two dactyls.

small amount of tissue in the ischium might account for the low percentage of claw transformation. Frozen claw tissue had no claw-transforming activity. The frozen tissues might not have contained sufficient morphogen for claw transformation or else claw transformation may be dependent upon a long-term or persistent induction process by the claw tissue. Stumps of both the third and fourth walking legs were able to be transformed into the claws. It remains to be seen if the first and the second walking legs can also be transformed.

Alteration of a walking leg into the likeness of a claw is a homeotic transformation (Bateson, 1894). Homeotic

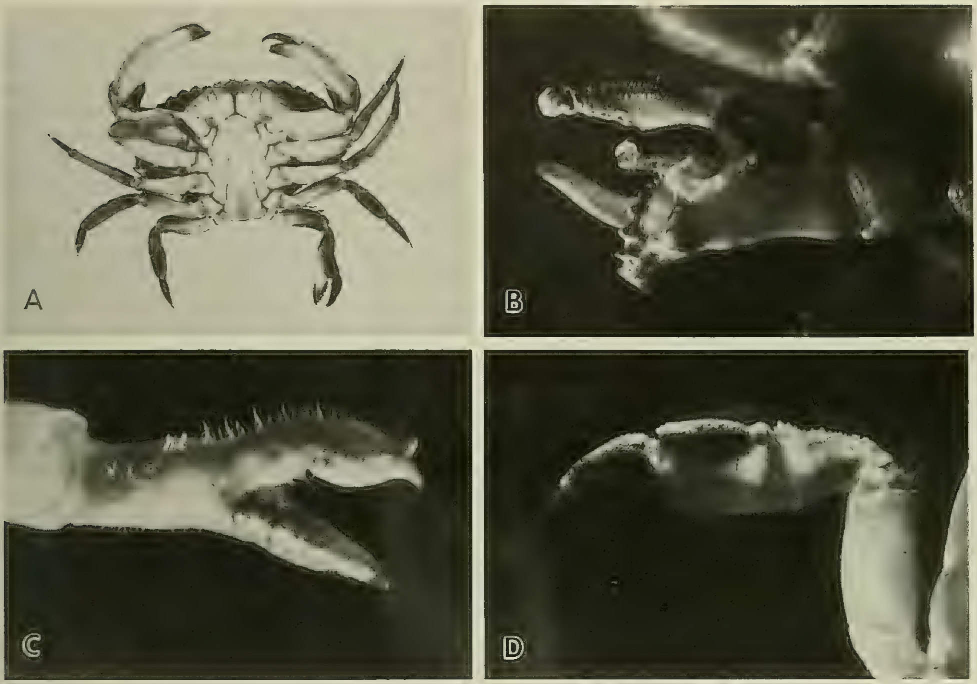

Figure 4. Regenerated limbs of the third or fourth walking leg in *Cancer anthonyi* after receiving the autotransplant of claw tissue. (A) Chimeric claw regenerated from the stump of the fourth left walking leg. (B) Regenerated walking leg with two pollexes and three dactyls. (C) Fork-shaped walking leg. (D) Regenerated walking leg with a clawlike dactyl.

transformation in nature is caused by a genetic defect of homeotic genes, such as *Drosophila HOM* or vertebrate *Hox* (reviewed by Krumlauf, 1994; Lawrence and Morata, 1994). Homeotic transformation can be experimentally induced with either morphogens or ectopic expression of *Hox* genes (Schneuwly *et al.,* 1987; Kessel and Gruss, 1990; Morgan *et al.,* 1992). The mechanism for the crab claw transformation is unknown. We propose three possible models: intercalary regeneration, morphogen, or change of *Hox* code.

Intercalary regeneration is a process that regenerates the missing part in some insects and amphibians (French *et al.,* 1976; Bryant *et al.,* 1981). It is possible that claw cells in the stump of the walking leg may regenerate the missing part to form a complete claw or else claw cells interact with walking-leg cells to regenerate a chimeric claw. This model is supported by the observation that autotransplanted frozen tissue had no activity. However, the result of contralateral autotransplantation does not favor this model since the positional value of the right and left limb is oriented in opposite directions. When the distal part of the right limb is transplanted to the proximal part of the left limb, it should regenerate three limbs from the stump according to the polar coordinate model (French *et al.,* 1976; Bryant *et al.,* 1981). In our contralateral autotransplantation experiments, we have never observed three limbs regenerating from the stump of a walking leg. Instead, a normal claw with the normal handedness can be regenerated. The intercalary regenerate model also does not fully explain the regeneration of abnormal walking legs, such as the leg with two pollexes and three dactyls. Together, these results suggest that the mechanism is not only a simple intercalary regeneration process. Some regulatory events, such as change of positional value and cell sorting, might be occurring during regeneration.

Because we autotransplanted claw tissue under the connective tissue septum of the walking leg stump, the claw tissue might secrete some morphogen that is able to induce claw transformation. Cohn *et al.* (1995) implanted a bead soaked with fibroblast growth factor into chick embryos. In some cases, they observed the induction of an extra leg or a leg–wing chimera if the bead was implanted at the wing level. When vitamin A was applied to amputated tail stumps of frog tadpoles, legs regenerated at the site of tail amputation (Mohanty-Hejmadi *et al.*, 1992; Maden, 1993; Mahapatra and Mohanty-Hejmadi, 1994). We propose that there is an induction process during claw transformation. The percentage and degree of claw transformation might depend upon the concentration of the morphogen secreted by the claw tissue. This model is supported by two experimental results. First, there is a range of claw transformation from a complete claw, to a chimeric claw, to a clawlike dactyl, to a Y-shaped walking leg, and finally to a fork-shaped walking leg. Second, the percentage of claw transformation in multiple claw-tissue autotransplantation is more than that of single-tissue autotransplantation. The effect of a morphogen might be through stimulation of cell proliferation and invagination at the apex of the blastema. Fork-shaped and Y-shaped walking legs might result from incomplete cell proliferation and invagination during claw morphogenesis. A limb with three dactyls and two pollexes might result from too much cell proliferation and invagination. Contralateral sham-operated crabs all regenerated normal walking legs, which suggests that autocrine or paracrine factors might be involved in claw transformation.

Alteration in the expression of *Hox* genes provides an alternative explanation of claw transformation. In visceral mesoderm of *Drosophila, Ultrabithorax* (*Ubx*), a *HOM-C* gene, is able to drive the expression of the *decapentaplegic* gene (*dpp*). *Dpp* encodes a protein that is like a secreted transforming growth factor, and *dpp* is able to control the expression of another *HOM-C* gene, *labial,* in the cells that are adjacent to it (Immeglück *et al.*, 1990; Reuter *et al.,* 1990). In the mouse embryo, ectopic expression of *Hoxb-8* causes duplication of the digits structure in the forelimb. *Sonic hedgehog* protein, fibroblast growth factor 4, and *Hoxd-11* are also ectopically activated at the anterior side of the limb bud (Charité *et al.,* 1994). It is possible that, during limb regeneration, those claw cells might express a set of active homeotic genes different from that of the walking leg cells. When claw cells were autotransplanted to the stump of the walking leg, the more anterior *Hox* genes might have been ectopically introduced into the stump of the walking leg. *Hox* genes in those claw cells might activate the expression of downstream genes and alter gene expression of neighboring cells. In this mechanism, the degree and percentage of claw transformation depend upon the percentage of cells in the stump that express the *Hox* code of the claw. The low percentage of claw transformation may be explained by the hypothesis that there is competition between the ectopically expressed genes and the genes normally expressed in a given segment. The cells in the stump of the walking leg express more posterior *Hox* genes than those of the transplanted claw cells. These more posterior genes may impose their developmental program in a dominant fashion over anterior *Hox* gene expression. This competition may lead to segmental transformations that are confined to the anterior body segment (Halder *et al.*, 1995). This phenomenon is called posterior prevalence (Lufkin *et al.*, 1991). In *Drosophila,* transformation of the more-posterior body segment toward the second thoracic segment, T2, is more difficult than transformation of the dorsal head capsule into T2. Further experiments could be conducted to determine if the claw or antenna is more easily transformed into a walking leg by autotransplantion of walking leg tissue.

Because most of the transformed claws have features of both the claw and the walking leg, we believe that both host and donor tissues are involved in regeneration of the transformed claw. However, we do not know the fate map of the grafted tissue in the regenerated limbs. Tissue transplantation between different species of *Cancer* crabs might be able to clarify this issue.

Acknowledgments

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Spawning, *in vitro* Maturation, and Changes in Oocyte Electrophysiology Induced by Serotonin in *Tivela stultorum*

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Abstract. Spawning was induced in both male and female Pismo clams by injecting 0.4 ml of 5 mM serotonin into the gonad. Spawned oocytes had already matured to metaphase I of meiosis or were undergoing germinal vesicle breakdown at the time of release. Prophase-arrested oocytes scraped from the ovaries of uninjected clams were induced to undergo germinal vesicle breakdown *in vitro* by incubating them with 0.2—20 μM serotonin in seawater; the former concentration was optimal. *In vitro* matured oocytes were fertilizable and developed to larvae, whereas sperm penetrated prophase-arrested oocytes without activating them. Fertilization was more successful in slightly alkaline seawater (pH 8.5). The electrical response of oocytes to serotonin was studied by means of intracellular microelectrode recording. Resting potentials of prophase-arrested oocytes were between −60 and −80 mV and there was no immediate electrical response to perfusion with serotonin. However, about 10–15 min later (before germinal vesicle breakdown), membrane potentials usually began to drift slowly in the positive direction (net change by 40 min + 9 ± 6.6 mV (SD; $n = 8$), whereas resting potentials of oocytes perfused with seawater alone usually drifted in the negative direction (−3 ± 6.1 mV; $n = 7$). A dramatic increase in input resistance was consistently observed in oocytes induced to mature with serotonin, probably due to the inactivation of K^+ channels, although this was not tested. Action potentials were always (7 out of 7 cases) present in maturing oocytes, but were detected only sometimes (7 of 14 cases) in prophase-arrested oocytes, presumably due to their lower input resistances.

Introduction

Tivela stultorum, the Pismo clam, is an important seafood in California and in Baja California, Mexico. However, populations have been seriously reduced by overcollecting (*e.g.,* Morris *et al.,* 1980). We were interested in developing a method for obtaining fertile gametes from the gonads to use for repopulation efforts. There are few previous studies of Pismo clam reproduction. Coe (1947) observed that normal development was not obtained when ovarian eggs were fertilized, but that spawned oocytes fertilized in the laboratory would develop to normal larvae. He mentioned that a spawning clam could stimulate neighbors to spawn, but that attempts to induce spawning in the laboratory were only "sometimes" successful. Fitch (1950) also observed neighbor-stimulated spawning, and stated he could induce spawning by raising the water temperature a few degrees. However, neither author presented data.

Because serotonin induces spawning, oocyte maturation, or both in a variety of bivalves (*e.g.,* Matsutani and Nomura, 1982; Gibbons and Castagna, 1984; Crawford *et al.,* 1986; Alcazar *et al.,* 1987; Osanai and Kuraishi, 1988; Hirai *et al.,* 1988; Ram *et al.,* 1993), we examined its effect on these processes in *Tivela.* Conditions for successful fertilization of matured ovarian oocytes were also explored. Furthermore, because serotonin is a common neurotransmitter, we studied its effects on electrical properties of the oocyte plasma membrane to better understand its mechanism of action during oocyte maturation.

Materials and Methods

Clams collected from San Quintín or San Ignacio, Baja California, Mexico, from July through November were

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maintained at 16°C in a seawater aquarium with a biological filter. For spawning tests, clams were placed individually in dishes containing 1.5 l of seawater for 1 h then injected intragonadally with 0.4 ml of serotonin (5-hydroxytryptamine creatinine sulfate complex, Sigma, St. Louis, MO), 5 m*M* in seawater with 5 m*M* Tris-HCl, pH 8. Controls were injected with 0.4 ml buffered seawater. Oocytes were also obtained directly from the ovaries by gently scraping their surfaces with a Pasteur pipet moistened with seawater. Oocyte suspensions in seawater were filtered through 120-μm nylon mesh (Small Parts, Inc., Miami Lakes, FL) to remove fragments of ovarian tissue, then poured into 60-μm mesh to trap the oocytes and wash them free of the milky contaminating body fluid. The oocytes were then washed several times in fresh seawater, and suspensions were maintained at 16°C. For some experiments vitelline coats were removed by vigorously pipeting oocyte suspensions through 60- or 120-μm mesh. Millipore-filtered (0.45 μm) natural seawater was used in all the experiments.

Induction of oocyte maturation *in vitro* was tested with various concentrations of serotonin in seawater (see Results). Germinal vesicle breakdown (GVBD) was assayed by ordinary light or phase-contrast microsopy in living or formaldehyde-fixed (5.5% in seawater) oocytes compressed under a coverslip, and progression to meiotic metaphase I was scored in fixed oocytes stained with aceto-orcein (method in Stephano and Gould, 1987) or bisbenzimide (method in Stephano and Gould, 1995).

Sperm were obtained by scraping the surface of testes as described above, then filtered through 60-μm mesh to remove tissue fragments. They were washed several times in seawater by centrifugation at 5000 × *g* (4°C, 5 min), and concentrated suspensions were stored on ice. Only batches with good motility (checked by dark-field microscopy) were used. Fertilization was carried out at either pH 8 (unmodified seawater) or 8.5 (adjusted with 0.5 *M* NaOH). Samples were fixed in 5.5% formaldehyde in seawater and stained for chromosomes and pronuclei with bisbenzimide.

Electrophysiological measurements were performed as described by Jaffe *et al.* (1979). Oocytes were immobilized against ridges scratched in the bottoms of plastic petri dishes and were penetrated with a single 30–60 MΩ electrode filled with 3 *M* KCl at 100× with a Wild (Wild Heerbrugg Instruments, Farmingdale, NY) stereomicroscope equipped with a water-cooled (16°C) stage. The dishes containing oocytes were grounded through an agar (0.5% in seawater) bridge and electrode resistances were subtracted with the bridge balance in the amplifier (BioDyne AM-2, Santa Monica, CA). In some experiments, serotonin solutions were added to the bath with a pipet (final concentration 2 μM). In other experiments solution changes were carried out in a perfusion chamber (total volume 0.4 ml) constructed with paraffin and containing a plastic baffle (see Jaffe *et al.*, 1979) to ensure that the entering solution flowed along the bottom of the dish where the oocytes were. Solutions (≥4 ml 2 μM serotonin or seawater alone) were added at one end of the chamber through a fine glass pipet connected to a syringe while excess liquid was removed through an aspirator pipet at the other end of the chamber.

Results

Spawning induction

After serotonin injection (see Methods), 10 of 21 clams (7 females and 3 males) spawned from 12 to 52 min later (average 32 min), whereas only 1 of 23 seawater-injected controls spawned (at approximately 100 min). Fourteen of the seawater-injected controls were then injected with serotonin 2–3 h later, and 5 of them (3 females and 2 males) spawned 38–85 min later. Spawning was preceded by characteristic behavior beginning about 15 min after injection: the shell valves opened periodically and then rapidly closed, expelling a jet of water from the exhalent siphon. This behavior was also observed in the control clam that spawned, but was not observed in any clams that did not spawn. Most of the spawned oocytes had already matured or were undergoing GVBD, and spermatozoa were motile within a few seconds of spawning.

Maturation in vitro

Oocytes removed from the ovaries without spawning induction were typically in meiotic prophase. At first they tended to be pear-shaped, then rounded up during incubation in seawater (Fig. 1). Fewer than 20% underwent GVBD during prolonged incubation in seawater (*e.g.*, "C", Fig. 2). The effects of incubating ovarian oocytes with varying concentrations of serotonin are illustrated in Figure 2. Concentrations inducing the highest percentage of GVBD were 0.2 to 20 μM, with the former concentration typically slightly better. At higher concentrations of serotonin, fewer oocytes matured (Fig. 2). Similar results were obtained in 15 additional experiments.

The procedure used for removing vitelline coats (VCs; see Methods) did not impair the ability of the oocytes to respond to serotonin. In four experiments with different egg batches, the percentage of GVBD was 82.5% ± 9.95% (SD; $n = 4$) in VC+ oocytes and 87.5% ± 13.23% in VC− oocytes exposed to 2 μM serotonin ($P > 0.2$, 2-tailed *t* test). The percentage of spontaneous GVBD following VC removal (15.25% ± 11.24%) was also not significantly different from that in intact oocytes (6.50% ± 4.51% ($P > 0.02$).

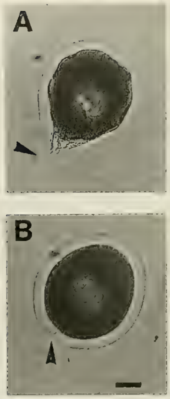

Figure 1. (A) Prophase-arrested *Tivela* oocyte recently removed from the ovary, showing the typical irregular shape and prominent "stalk" (arrow) at the vegetal pole where the oocyte was presumably attached to the ovarian wall. The vitelline coat and germinal vesicle are also clearly visible in this ordinary light micrograph. (B) An immature oocyte after it has rounded out during incubation in seawater. The cytoplasm in the stalk has retracted, leaving a scar in the vitelline coat (arrow). The germinal vesicle is still intact. Bar, 20 μm.

Fertilization of oocytes matured in vitro

When prophase-arrested oocytes removed from the ovary without exposure to serotonin were inseminated, bisbenzimmide staining revealed that sperm entered but the oocytes failed to respond: GVBD did not occur and development was not initiated. However, following maturation *in vitro* with serotonin, fertilization induced normal development. When fertilization was carried out at pH 8, rather high sperm concentrations were required; therefore we tried fertilizing oocytes at a higher pH, since in some species sperm undergo the acrosome reaction more readily when the pH is raised (*e.g.*, Decker *et al.*, 1976). Although we did not investigate the mechanism with *Tivela*, this strategy was successful. In two experiments with insemination at pH 8.5, development to normal blastulae occurred in 38% and 14% of mature oocytes inseminated with 100 sperm/oocyte, in 73% and 70% inseminated with 1000 sperm/oocyte and in 72% and 84% inseminated with 10,000 sperm/oocyte. The corresponding percentages for oocytes inseminated at pH 8 were 1.5 and 2.3, 47 and 35, and 65 and 52 ($n > 130$ for each sample). Development to "D" larvae was common although development beyond blastula was not quantitated. Sperm were obtained directly from the gonad and did not require serotonin treatment to fertilize eggs.

Electrophysiological response to serotonin

Resting potentials of prophase-arrested oocytes were typically between −60 and −80 mV, with no significant difference between intact (−68 ± 8 mV SD; $n = 8$) and denuded (−69 ± 10 mV; $n = 10$) oocytes. Upon application of serotonin, no immediate electrical response was observed. However, about 10 to 15 min later, resting potentials usually began to drift slowly in the positive direction. By about 40 min after serotonin application, the potential had drifted +17, +15, +9, and 0 mV in 4 oocytes with vitelline coats, and +15, +9, +7, and 0 mV in 4 denuded oocytes (mean +9 ± 6.6 mV). All of these oocytes had undergone GVBD. The positive drift was not observed in oocytes that were treated with seawater alone; in fact the resting potentials of these oocytes tended to drift to more negative values with time (−13, −6, −5, −2, −2, 0, and +7 in 7 oocytes with and without vitelline coats; mean −3 ± 6.1). This difference between the two treatments was significant ($P < 0.01$)

A dramatic change consistently observed in oocytes induced to mature with serotonin was an increase in input resistance. An example is shown in Figure 3. The I-V relation for this oocyte shortly after electrode penetration was almost a straight line with a slope resistance of 35 MΩ from −0.075 to −0.33 nA ($r = 0.992$; Fig. 3A, B). Ten minutes later, after perfusion with seawater alone, the slope resistance had decreased to about 3 MΩ (−0.4 to +0.4 nA, $r = 0.958$; Fig. 3C), and this situation persisted through 50 min, when the oocyte was perfused with serotonin. By 15 min after serotonin perfusion, the resistance had increased markedly (83 MΩ for the linear segment from −0.075 to −0.33 nA shown in Fig. 3A; $r = 0.998$), and an action potential appeared as an "on" response to a +0.035 nA current pulse (Fig. 3D), and as an "off" response following a −0.33 nA pulse (Fig. 3E). GVBD occured at 19 min after serotonin perfusion.

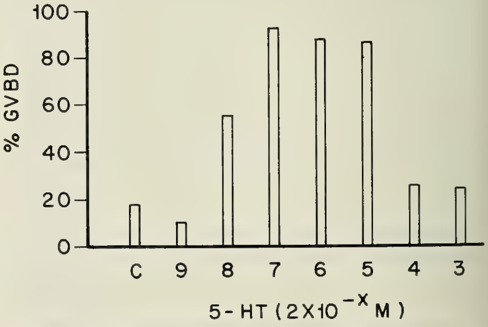

Figure 2. A typical dose-response curve for the induction of maturation (GVBD = germinal vesicle breakdown) by serotonin (5-HT). The optimum concentration is 200 n*M*, and high concentrations (≥0.2 m*M*) are much less effective. C = control, with a low percentage of spontaneous activation.

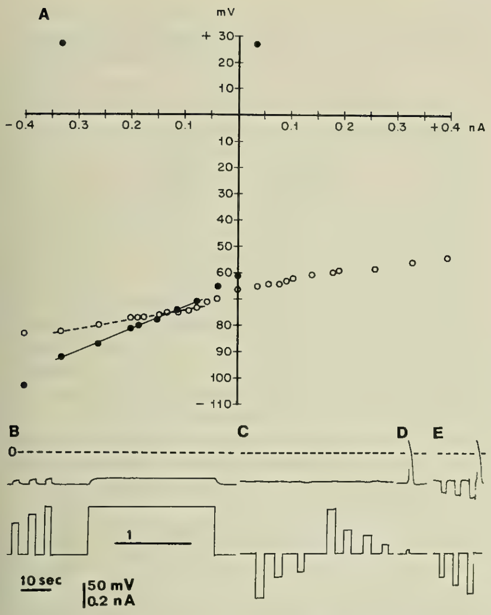

Figure 3. (A) Voltage responses to current pulses applied to an oocyte before (O) and after (●) serotonin treatment. The data shown by (O) were obtained 7–9 min after electrode penetration, serotonin was added by perfusion at 50 min, and the data shown by (●) were obtained 15 min later. GVBD occurred 19 min after serotonin addition. Slope resistances were 35 MΩ (−0.075 to −0.33 nA) before serotonin, and 83 MΩ (−0.075 to −0.33 nA) after serotonin. (B)–(E). Samples of voltage (upper) and current (lower) records at various times during the experiment: (B) 9 min after electrode penetration; (C) 25 min later, after seawater perfusion (see text); (D) and (E) 16 min after serotonin perfusion—(D) shows the action potential "on" response to +0.035 nA and (E) shows the "off" response to −0.33 nA.

The resistance increase occurred in every oocyte exposed to serotonin. For example, in 4 other oocytes with VCs, the slope resistances rose from 7, 18, 135, and 179 MΩ before serotonin, to 43, 87, 286, and 284 MΩ afterwards. Similar results were obtained with denuded oocytes: input resistances rose in 6 of 6 after serotonin addition and declined in 4 of 4 after seawater addition. As mentioned above, resistances tended to decline after seawater perfusion.

The resistance increase began after a short delay. Resistances were still low in 3 of 3 oocytes tested from 1 to 1.5 min after serotonin perfusion was completed, then a substantial increase was noticeable at 2 min in one of these oocytes (the others were not tested at this time). GVBD occurred after the resistance increase in 4 of 4 oocytes for which the timing was observed carefully.

In some, but not all, starfish species examined, capacitance measurements indicate a substantial loss of membrane surface area during maturation (Moody and Bosma, 1985; Simoncini and Moody, 1990). We did not investigate capacitance changes in *Tivela* oocytes, so no conclusion is possible. However, an "anecdotal" calculation based on time constant and resistance measurements before and after serotonin treatment in the oocyte of Figure 3, revealed essentially no change: at 9 min after electrode penetration a current pulse of −0.4 nA produced a voltage change of −7 mV with a time constant of 0.032 s ($C = 1.8 \times 10^{-9}$ F), and at 18 min after serotonin perfusion −0.078 nA produced a −9 mV shift with a time constant of 0.2 s ($C = 1.7 \times 10^{-9}$ F).

As mentioned above, an action potential is present in *Tivela* oocytes. The threshold potential was −45 to −50 mV and peak potentials ranged from +20 to +35 mV; a typical example is shown in Figure 4A. Action potentials were always detected after serotonin treatment (7 of 7 oocytes), but sometimes (7 of 14 oocytes) were not observed in prophase-arrested oocytes, presumably be-

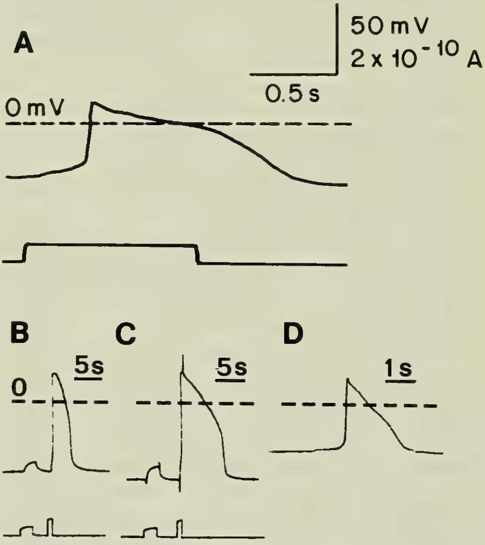

Figure 4. (A) Typical action potential elicited by a current pulse. Recording is from an oocyte 18 min after serotonin perfusion (GVBD occurred at about 20 min). The resting potential was −58 mV, the voltage threshold was −45 mV, and the peak amplitude was +21 mV. (B) and (C) Action potentials from another oocyte 2 min before (B) and 10 min after (C) serotonin perfusion. Although current pulses were identical, the action potential in the maturing oocyte (at about the time of GVBD) was of longer duration. (D) One of a train of 52 spontaneous action potentials fired by an oocyte following serotonin treatment (see text).

cause membrane resistances were so low that the applied current pulses failed to depolarize the membrane to the voltage threshold. For example in the oocyte of Figure 3, an action potential occurred as an "off" response to negative current injection at 3 min after penetration, then a spontaneous action potential occurred as the resting potential passed below −45 mV during recovery from electrode damage (not shown). Subsequently, action potentials were not detected with the current pulses used until after serotonin treatment (Fig. 3). When there was a robust action potential in an oocyte before serotonin treatment (Fig. 4B), it became even more prominent at about the time of GVBD (Fig. 4C). Later, more current was required to elicit the action potential and the duration decreased (not shown). Similar results were observed in the oocyte of Figure 3. By 20 min after GVBD the resting potential had drifted to −50 mV, the slope resistance had declined to 47 MΩ, and +0.11 nA was required to elicit an action potential with a peak amplitude of only −2 mV. These results suggest that oocyte excitability may be maximal at about the time of GVBD, but more data are required for a conclusion.

Sometimes spontaneous action potentials also occurred as the membrane potential crossed the threshold voltage during its positive shift in response to serotonin. In one oocyte, as the resting potential drifted to −50 mV about 25 min after serotonin addition, spontaneous action potentials of about 2-s duration began to recur at intervals ranging from 7 to 20 s; a total of 52 were recorded during 9.6 min until the electrode unfortunately came out. Figure 4D shows a typical action potential from this train.

These results show that serotonin causes a decrease in the oocyte plasma membrane conductance accompanied by a small positive shift in the resting potential and increased membrane excitability. The possible mechanisms involved and biological significance are discussed below.

Discussion

The above results show that serotonin can induce spawning and oocyte maturation in Pismo clams and that maturation is acompanied by changes in the electrical properties of the oocyte plasma membrane so that the input resistance is increased and an action potential is more readily elicited.

As mentioned in the introduction, injection of serotonin into the gonad or other tissue (adductor muscle, foot, etc.) has been shown to induce spawning in a number of bivalve molluscs. Valve opening and closing prior to spawning was also observed by Galtsoff (1938) in oysters. Matsutani and Nomura (1982) found that although scallops (*Patinopecten yessoensis*) of both sexes spawned following serotonin injection, males responded to lower doses ($\geq 2\ \mu M$) than females (who required ≥ 0.2 mM), and tended to spawn sooner. Both sexes of the freshwater bivalve *Dreissena polymorpha* also spawned in response to serotonin (Ram *et al.,* 1993). On the other hand, in the study of Gibbons and Castagna (1984), males of various species were observed to spawn more frequently than females following serotonin (2 mM) injection. Our results show that *Tivela* females respond readily to serotonin. In fact, more females than males spawned, but larger numbers of animals are required to establish whether there are any differences in sensitivity between the two sexes.

Serotonin has been detected in tissues of various marine bivalves (Stefano and Catapane, 1977; Smith, 1982; Matsutani and Nomura, 1984; Kadam and Koide, 1989; Martínez and Rivera, 1994). However, whether serotonin is a natural trigger during the spawning process, and if so, whether it acts directly on the oocyte, on somatic cells in the ovary, or on neurons is still unknown. In *Tivela,* ovarian oocytes must have serotonin receptors because oocytes with no follicle cells attached respond to serotonin in seawater. Furthermore, the optimal concentration of serotonin for GVBD and maturation to metaphase I is low (200 nM), supporting the hypothesis that serotonin is a natural trigger. However, it remains to be shown that serotonin is released in the ovary at the time of spawning, and that blocking this release or inhibiting serotonin binding to oocytes in the ovary prevents maturation.

Serotonin has also been shown to induce GVBD followed by arrest in metaphase I in immature oocytes removed from the ovary of the oyster *Crassostrea gigas* (Osanai and Kuraishi, 1988) and the clam *Ruditapes philippinarum* (Osanai and Kuraishi, 1988; Guerrier *et al.,* 1993). (These oocytes also have no accessory cells attached.) However, higher concentrations of serotonin are required for maximum percentages of GVBD: on the order of 10 μM in *Crassostrea,* and 1 μM in *Ruditapes* (Osanai and Kuraishi, 1988; Guerrier *et al.,* 1993). As in *Tivela,* prophase-stage oocytes of *Ruditapes* do not develop when fertilized (Osanai and Kuraishi, 1988; Guerrier *et al.,* 1993). Oyster oocytes, on the other hand, can be fertilized and will complete meiosis and develop normally when they are inseminated at any stage from prophase to metaphase I (*e.g.,* Imai *et al.,* 1950). In the clam *Spisula solidissima,* GVBD in isolated oocytes is also induced by serotonin (10 μM), but the biological significance is unclear because the treated oocytes (which complete meiosis and form a pronucleus instead of arresting at metaphase I) are reported to be unfertilizable (Hirai *et al.,* 1984, 1988). Furthermore, *Spisula* oocytes with intact germinal vesicles are readily fertilized and will develop (*e.g.,* Allen, 1953); when spawning is induced by injecting clams with serotonin, most of the spawned oocytes have intact germinal vesicles and undergo GVBD after fertilization (Hirai *et al.,* 1988). Unlike *Tivela* oocytes, those of *Spisula* were reported to be less responsive to serotonin following VC removal (Krantic *et al.,* 1993). However, this difference could be due to damage, because the *Spisula* VCs were removed by a rather harsh treatment, 1 *M* glycerol in 0.2 *M* phosphate buffer.

In summary, although in all of these species serotonin may experimentally induce adults to spawn and isolated oocytes to undergo GVBD, there are significant variations in both sensitivity and response, and the roles of serotonin in the natural induction of these processes remain to be elucidated.

How does serotonin induce the reinitiation of meiosis and the electrical changes in the *Tivela* oocyte membrane? A variety of serotonin receptors have been identified and characterized in neurons and other somatic cells of both vertebrates and invertebrates (*e.g.,* reviews by Hen, 1992; Frazer and Hensler, 1994). Many are coupled to G-proteins and others are ligand-gated ion channels (*e.g.,* Hen, 1992; Frazer and Hensler, 1994; Shih *et al.,* 1995). In *Ruditapes* oocytes, a transient increase in inositol-3,4,5-triphosphate (IP3) peaked at 3 min after serotonin application, and GTP and GTP γS induced GVBD in a majority of injected oocytes (Gobet *et al.,* 1994), consistent with, but not proving, the involvement of a G-protein-linked receptor.

In our studies there was no immediate electrical response to serotonin, suggesting that the hormone may not act *via* a ligand-gated channel. Rather, a decreased conductance developed slowly. Although we did not investigate which channels were being inactivated to cause the conductance decrease, the most likely candidates are K^+ channels, as shown in starfish oocytes where maturation is accompanied by decreased membrane conductance (Shen and Steinhardt, 1976; Miyazaki, 1979; Moody and Lansman, 1983; Simoncini and Moody, 1990). Voltage-clamp analysis showed that the decrease in conductance was primarily due to inactivation of inwardly rectifying K^+ channels (so-called because they are activated by hyperpolarizing rather than depolarizing membrane potentials) (Moody and Lansman, 1983; Moody and Bosma, 1985; Simoncini and Moody, 1990). Ca^{2+} action potentials are present in immature starfish oocytes, and with the decreased conductance, less current was required to elicit them and their duration was longer (Shen and Steinhardt, 1976; Moody and Lansman, 1983; Moody and Bosma, 1985; Simoncini and Moody, 1990). The action potentials in *Tivela* oocytes responded in the same way to the decreased conductance during maturation (see Fig. 4).

In some other species, the increased membrane resistance and more prominent action potentials in oocytes at the stage when they are fertilized strengthen the electrical polyspermy block. Examples among marine invertebrates are in sea urchin (Jaffe, 1976; Jaffe and Robinson, 1978), starfish (Miyazaki and Hirai, 1979; Miyazaki, 1979), and the marine worm *Urechis* (Gould-Somero *et al.,* 1979; Holland and Gould-Somero, 1981). Although *Tivela* might be expected to have an electrical polyspermy block, this remains to be shown.

Whether trains of spontaneous action potentials (see Fig. 4D and related text) might also occur during *in vivo* maturation of *Tivela* oocytes is an intriguing question. For example, if these are Ca^{2+} action potentials they might cause increases in intracellular Ca^{2+} that could play a role in inducing maturation. Indeed, Schlichter (1983a, b) observed that maturing *Rana pipiens* oocytes fired trains of spontaneous Na^+ action potentials during the period from metaphase I to 1st polar body formation, and when these were prevented, maturation was delayed.

Acknowledgments

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Chemical Mediation of Sperm Activity and Longevity in the Solitary Ascidians *Ciona intestinalis* and *Ascidiella aspersa*

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Abstract. Sperm chemotaxis to compounds originating from eggs is common in solitary ascidians. This chemotactic response is associated with an increase in the activity level of the sperm. Here we examine the hypothesis that the period of sperm viability is reduced by the stimulation of increased activity by compounds associated with the eggs of solitary ascidians. The activity level and longevity of sperm from two species of solitary ascidian, *Ciona intestinalis* and *Ascidiella aspersa,* were measured after incubation in water that had been stored with homospecific eggs. Our results showed that the proportion of active sperm increased in homospecific egg water. This increase corresponded to a sharp decline in sperm longevity, which suggests a causal link between sperm activity and longevity. We suggest that sperm energy reserves are conserved in the absence of compounds associated with homospecific eggs, and that this may have significant ecological consequences for these organisms. Cross-reactivity in the chemotactic response of sperm from one species to the chemoattractant of another is common in ascidians, and increased sperm activity is associated with this response. Therefore, we investigated cross-reactivity in the activation of *C. intestinalis* and *A. aspersa* sperm to heterospecific egg water. Our results showed that *A. aspersa* sperm were activated by the egg water of *C. intestinalis,* resulting in a decline in sperm viability, but that this response was not reciprocated between the sperm of *A. aspersa* and the egg water of *C. intestinalis.* This suggests either that sperm of *A. aspersa* respond to compounds associated with the eggs with a higher degree of specificity than *C. intestinalis* sperm, or that compounds associated with *A. aspersa* eggs have more generalized activity than those of *C. intestinalis.*

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Introduction

Interest in the importance of fertilization kinetics and fertilization success in free-spawning marine invertebrates has recently increased (*e.g.,* Pennington, 1985; Denny and Shibata, 1989; Levitan, 1991; Levitan *et al.,* 1992). Selective pressure to maximize the probability of gamete contact, and hence fertilization success, is widely assumed to be the reason for the evolution of synchronous spawning in many species (*e.g.,* Pennington, 1985; Pearse *et al.,* 1988; Levitan, 1991) and one of the pressures for aggregated field distributions exhibited by many sessile marine invertebrates (*e.g.,* Levitan, 1991; Levitan *et al.,* 1992). Perhaps the strongest evidence of the importance of fertilization success as a selective pressure comes from the evolution of sperm chemotaxis in many marine phyla.

Sperm chemotaxis is the directional orientation and swimming of sperm towards compounds originating from eggs and reproductive structures. Sperm chemotaxis has been demonstrated in species from four marine invertebrate phyla: Cnidaria (Miller, 1966, 1978, 1979); Mollusca (Miller, 1977); Urochordata (Miller, 1975); and Echinodermata (Ward *et al.,* 1985; Miller, 1985). The adaptive significance of sperm chemotaxis is believed to lie in the increased probability of gamete contact, even when gamete concentration is low (Miller, 1982).

Few attempts have been made to isolate and characterize the compounds that stimulate sperm chemotaxis, and much of the work that has been done has centered on sea urchins (*e.g.,* Kopf *et al.,* 1979; Hansbrough and Garbers, 1981; Suzuki and Garbers, 1984). Resact, a peptide isolated from the jelly coat of the sea urchin egg, has been shown to induce a species-specific chemotactic response in sea urchin sperm (Ward *et al.,* 1985). Resact and another peptide isolated from sea urchin egg jelly coats, speract, have also been shown to increase the activity level and respiration rate of sea urchin sperm on a species-specific basis (Suzuki and Garbers, 1984). Thus, increases in both sperm activity and respiration are characteristic of the chemotactic response of sea urchin sperm.

Sperm chemotaxis is widespread amongst solitary ascidians (Miller, 1982). The compounds that induce sperm chemotaxis in ascidians have not been isolated and characterized. However, increased sperm activity also appears to be associated with the chemotactic response in these organisms. For example, Minganti (1951) reports that eggs, or water that has contained homospecific eggs, activate nonmotile sperm of *Ciona intestinalis.* Miller (1974) also reports that activity can be stimulated in *C. intestinalis* sperm by the injection of water that has contained homospecific eggs, and that this water also induces chemotactic behavior in the sperm (Miller, 1975). Similarly, we have observed an increase in the level of activity of sperm from two species of solitary ascidian, *C. intestinalis* and *Ascidiella aspersa,* upon exposure to water that has contained homospecific eggs.

We suggest that the induction of increased sperm activity with exposure to compounds associated with the egg may reduce sperm longevity by depleting the energy reserves of the sperm. We tested this hypothesis with two species of free-spawning solitary ascidian, *Ciona intestinalis* and *Ascidiella aspersa.* Sperm activation and chemotaxis in response to compounds originating from homospecific eggs have been demonstrated in *C. intestinalis* (Miller, 1975), and sperm activation and agglutination have been reported in *A. aspersa* upon exposure to water that has contained homospecific eggs (Minganti, 1951). We measured levels of sperm activity and longevity from both species in response to water that had been incubated with homospecific eggs ("egg water").

Chemotactic cross-reactivity between the sperm of one species and the eggs or egg extracts of another appears to be common in ascidians (see data in Miller, 1982). Because increased sperm activity appears to be associated with the chemotactic response, we predicted that any response in sperm activity and longevity to egg water would be interspecific between *C. intestinalis* and *A. aspersa.* We investigated this hypothesis by exposing the sperm of each species to the egg water of the other and measuring sperm activity and longevity.

Our results indicate that sperm of these species display increased activity to homospecific egg water, and that this results in a significant decline in the period of sperm longevity. We found that activity and longevity of sperm of *C. intestinalis* displayed cross-reactivity to *A. aspersa* egg water but that cross-reactivity of *A. aspersa* sperm to *C. intestinalis* egg water did not occur. We propose that the activation of nonmotile sperm by compounds associated with the eggs may provide a mechanism by which the energy reserves of a sperm can be conserved in the absence of eggs, thereby maintaining sperm viability for extended periods.

Materials and Methods

Collection of adults and gametes

Adult *Ciona intestinalis* and *Ascidiella aspersa* were collected from pier pilings at North Haven marina, Adelaide, South Australia, and held in a laboratory aquarium system. As these organisms are hermaphrodites, both sperm and eggs were obtained from individual animals by dissecting the outer tunic and tissues to expose the gonoducts. The exposed gonoducts were flushed with MBL artificial seawater (ASW) (Strathmann, 1987) to remove acidic body fluids, which may affect gamete viability. Eggs and sperm were then extracted with a pipette inserted into the respective gonoduct. Sperm were suspended in filtered (0.22 μm) ASW (35‰) at ambient field temperature (18°C). Eggs were rinsed with ASW to remove the viscous mucus, which adheres to all eggs, before being suspended in ASW at 18°C.

Sperm longevity

The longevity of sperm from *Ciona intestinalis* and *Ascidiella aspersa* was measured as a function of ability to fertilize freshly extracted eggs at regular time intervals after exposure to three treatments: *A. aspersa* egg-water solution (AEW), *C. intestinalis* egg-water solution (CEW), and artificial seawater control (ASW).

Egg-water solutions were made by incubating 1000 eggs in 10 ml of ASW for 24 h at 18°C. Five replicate egg-water solutions were made from the eggs of five individuals from each species. These solutions were filtered (0.22 μm) to remove particulate matter. The ASW control was stored and filtered in the same manner as the egg-water solutions. Large petri dishes (90-mm diameter) were used for storage of all solutions to facilitate adequate gaseous exchange. The pH of all solutions was measured at the end of the 24-h incubation period.

For each species, five sperm solutions at a concentration of about 10,000 sperm/μl were made from five individuals. A 5-ml aliquot from each of these solutions was pipetted into separate petri dishes containing 50 ml of each treatment solution (five replicates for each of the three treatments). The resulting sperm concentration in each replicate was about 1000 sperm/μl. Solutions were incubated

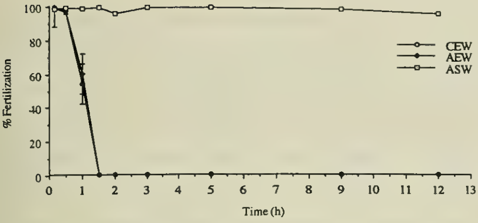

Figure 1. Viability of *Ciona intestinalis* sperm measured as a function of its ability to fertilize freshly extracted eggs over time with incubation in treatments. Each datum point represents the mean (±SE) proportion of eggs fertilized by incubated sperm from five replicates. Symbols for different treatments are identified in the figure. Treatments: CEW, solution of artificial seawater incubated with *C. intestinalis* eggs; AEW, solution of artificial seawater incubated with *A. aspersa* eggs; ASW, artificial seawater control.

at 18°C. After 10 min, 30 min, 1 h, 1.5, 2, 3, 5, 9, and 12 h, a 500-μl aliquot from each replicate was added to 35-mm petri dishes containing 4 ml of ASW and 100 freshly extracted eggs from one individual. Thus, final sperm concentration was 125 sperm/μl. After 2 h the proportion of eggs that were fertilized was recorded. Fertilization was recorded if the egg had divided or begun to divide.

Sperm activity

To determine whether egg-water solutions increased the activity level of sperm, we measured the proportion of active sperm in each of the treatments (ASW, CEW, and AEW) over time. At the same time intervals used in the sperm longevity trials, a drop of each treatment solution (*i.e.,* the same solutions used in the sperm longevity trial) was pipetted onto a depression slide. Images of the sperm were recorded onto S-VHS video tape at 200× magnification under an Olympus BHS compound microscope with the focal plane set to the center of the slide. The proportion of sperm actively beating their tails over 10 s of video image was determined.

Statistical analysis

All data were arcsine transformed prior to analysis (Zar, 1984). Sperm longevity and activity data were analyzed by two-way repeated measures ANOVA. Significance was determined at the 5% level. Locations of any significant differences were determined by Tukey's test (Zar, 1984).

Results

Eggs of both species were viable after the 24-h incubation period. The pH of all treatment solutions after the incubation period was between 7.6 and 7.8 and therefore within the range of normal seawater.

Sperm longevity

Sperm from *Ciona intestinalis* were viable throughout the 12-h period of the experiment in the control (ASW) (Fig. 1). In contrast, the longevity of *C. intestinalis* sperm was greatly reduced when incubated with either homospecific or heterospecific egg water (CEW and AEW respectively, Fig. 1). Two-way repeated measures ANOVA showed a significant difference in the longevity of sperm between treatments over time ($F = 644$, $P < 0.0001$). The viability of *C. intestinalis* sperm was high in all treatments from initiation of the experiment to 30 min after its beginning. Viability of *C. intestinalis* sperm then declined rapidly in both the *C. intestinalis* egg water (CEW) and the *A. aspersa* egg water (AEW) (Fig. 1). Tukey's test indicated that, after 1 h incubation in the CEW and AEW treatments, the viability of *C. intestinalis* sperm had declined significantly below the level seen in the ASW control treatment (CEW: $q = 110.63$, $P < 0.0001$; AEW: $q = 91.26$, $P < 0.0001$). After 1.5-h incubation in the CEW and AEW treatments, *C. intestinalis* sperm were no longer capable of fertilizing eggs (Fig. 1).

Sperm from *Ascidiella aspersa* remained viable throughout the period of the experiment in both the control (ASW) and heterospecific (CEW) treatments (Fig. 2); however, the longevity of *A. aspersa* sperm was greatly reduced in the homospecific (AEW) egg-water treatment (Fig. 2). Again, two-way repeated measures ANOVA indicated that significant differences existed in sperm longevity between treatments over time ($F = 154.64$, $P < 0.0001$). Viability of *A. aspersa* sperm was high in all treatments from initiation of the experiment up to 1 h (Fig. 2). Sperm viability then declined rapidly in the AEW treatment and was significantly lower than in the CEW and ASW treatments after 1.5 h incubation ($q = 105$, P

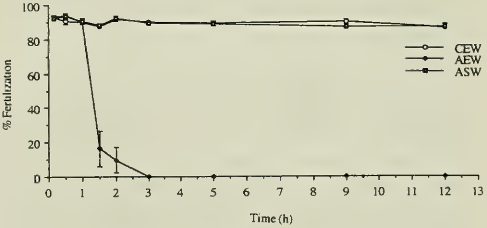

Figure 2. Viability of *Ascidiella apersa* sperm measured as a function of its ability to fertilize freshly extracted eggs over time with incubation in treatments. Refer to Fig. 1 for an explanation of treatments.

< 0.0001). *A. aspersa* sperm were incapable of fertilizing eggs after 3 h incubation in homospecific egg water (Fig. 2).

Sperm activity

The proportion of active *Ciona intestinalis* sperm was greater in the homospecific (CEW) and heterospecific (AEW) egg-water treatments than in the control (ASW) treatment up to 1 h after initiation of the experiment (Fig. 3). Analysis of variance showed that the proportion of active sperm differed significantly between treatments over time ($F = 296.03$, $P < 0.0001$). Tukey's test showed that, after 1 h, the proportion of active *C. intestinalis* sperm in the CEW and AEW treatments was significantly higher than in the ASW control treatment ($q = 91.26$, $P < 0.0001$). No significant difference in the proportion of active *C. intestinalis* sperm was found between the CEW and AEW treatments at this time ($q = 3.87$, $P > 0.05$). After 1.5 h incubation, the proportion of active *C. intestinalis* sperm in the CEW and AEW treatments declined to a level that was significantly lower than in the ASW control ($q = 108.63$, $P < 0.0001$) (Fig. 3). Sperm of *C. intestinalis* in the CEW and AEW treatments showed no activity after 3 h. Some sperm were noted to twitch their tails periodically; however, this was not recorded as representing activity. *C. intestinalis* sperm in the ASW control treatment remained active for the duration of the experiment (12 h), although the proportion of active sperm declined steadily between 3 h and 12 h (Fig. 3).

More *Ascidiella aspersa* sperm were active in the homospecific (AEW) treatment than in the heterospecific (CEW) and ASW control treatments during the first 1.5 h of the experiment (Fig. 4). Analysis of variance indicated that there was a significant difference between the proportion of active sperm in treatments over time ($F = 223.82$, $P < 0.0001$). Tukey's test showed that, after 1.5 h incubation, the proportion of *A. aspersa* sperm active was significantly greater in homospecific egg water (AEW) than in the CEW and ASW treatments ($q = 96.4$, $P < 0.001$). Activity of *A. aspersa* sperm declined rapidly after 1.5 h in homospecific egg water (AEW) (Fig. 4): after 2 h incubation the proportion of *A. aspersa* sperm active in the AEW treatment was lower than that in either the CEW or ASW treatments ($q = 20.63$, $P < 0.0001$). Significant differences were also apparent between the CEW and ASW treatments at this time ($q = 24.74$, $P < 0.0001$). Sperm activity in the AEW treatment declined rapidly such that no activity was recorded after 3 h. In contrast, *A. aspersa* sperm in the CEW and ASW treatments remained active throughout the experiment, although the proportion of active sperm declined gradually from 3 h to 12 h (Fig. 4).

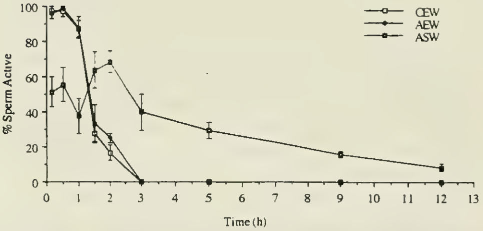

Figure 3. Proportion of active *Ciona intestinalis* sperm in treatments over time. Each datum point represents the mean (±SE) proportion of active sperm in five replicates. Symbols for different treatments are identified in the figure. Treatments: CEW, solution of artificial seawater incubated with *C. intestinalis* eggs; AEW, solution of artificial seawater incubated with *A. aspersa* eggs; ASW, artificial seawater control.

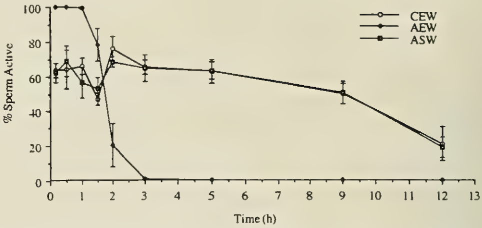

Figure 4. Proportion of active *Ascidiella aspersa* sperm in treatments over time. Each datum point represents the mean (±SE) proportion of active sperm in five replicates. Symbols for different treatments are identified in the figure. Refer to Fig. 1 for an explanation of treatments.

Discussion

Sperm activity and longevity

Sperm of both *Ciona intestinalis* and *Ascidiella aspersa* were viable for at least 12 h in artificial seawater (ASW) (Figs. 1, 2). However, sperm longevity declined rapidly when sperm were incubated with homospecific egg water (Figs. 1, 2). This reduction in longevity corresponded with an increase in sperm activity (Figs. 3, 4). We therefore suggest a causal link between sperm activity and sperm longevity. These results support our initial hypothesis that ascidian sperm are activated by homospecific egg water and that this activation results in a reduction in sperm longevity and hence fertilization ability.

Because cross-reactivity in the chemotactic response of solitary ascidian sperm is common (Miller, 1982) and increased sperm activity is associated with this response, we predicted that any response in sperm activity and longevity to egg water would be interspecific between *C. intestinalis* and *A. aspersa*. Contrary to this prediction, our data show that the response of sperm to heterospecific egg water was not reciprocated between *C. intestinalis* and *A. aspersa.* Sperm of *C. intestinalis* were activated in *A. aspersa* egg water (AEW) (Fig. 1), whereas *A. aspersa* sperm were not activated in *C. intestinalis* egg water (CEW) (Fig. 2). These data suggest that *A. aspersa* sperm respond to compounds associated with the eggs with a higher degree of specificity than do *C. intestinalis* sperm. The compounds that stimulate ascidian sperm activity and taxis have not been characterized, so the basis of this specificity is unknown. Because the response of *C. intestinalis* and *A. aspersa* sperm to heterospecific egg water was not the same, studies to determine the chemical structure of the compounds responsible and their mode of action in these species may provide valuable insight into the mechanism of species specificity of both sperm activation and taxis.

Our results show that the proportion of active sperm declined over time in the control treatments. For example, at 12 h the proportion of *C. intestinalis* sperm that remained active had declined to about 10% of the activity observed at 10 min (Fig. 3). Despite this reduction in the proportion of active sperm, fertilization success remained at 100% (Fig. 1). The sperm concentration used in the fertilization experiment reported here was 125 sperm/μl and the exposure time of the eggs to the sperm was 2 h. If only 10% of the sperm remained active after 12 h, the concentration of viable sperm that eggs were exposed to would be about 12 sperm/μl. Subsequent experiments conducted to determine the relationship between sperm concentration and fertilization success in these species indicate that concentrations of <10 sperm/μl with a relatively short exposure time are sufficient to result in 100% fertilization success (Bolton and Havenhand, unpub. data). The high rates of fertilization success found in the control treatments are therefore most likely to be the result of a saturating sperm concentration and extended exposure period.

Sperm were incubated in the egg water and control treatments at a concentration of 1000 sperm/μl. Previous studies have indicated that the longevity of marine invertebrate sperm is highly dependant on the concentration at which they are stored. For example, the longevity of sea urchin sperm is extended when sperm are stored at high concentrations (Pennington, 1985). It could therefore be argued that the extended longevity of sperm found in the control (ASW) treatments was due to the concentration at which they were incubated. This is relevant when comparing our results from the control treatments with those from other studies. However, although sperm concentration may have had some effect on the absolute period of sperm longevity, the findings of this work were not affected because sperm concentrations in all treatments were equal and therefore internally consistent.

The peptides speract and resact, which have been isolated from the jelly coat of sea urchin eggs and shown to stimulate the activity and respiration of sea urchin sperm, are most active at acidic pH levels and only slightly active at the alkaline pH of seawater (7.6–8.0) (Hansborough and Garbers, 1981; Suzuki *et al.,* 1981; Garbers *et al.,* 1982). The biological activity of these compounds has therefore been questioned (Holland and Cross, 1983; Suzuki and Garbers, 1984). In the experiments conducted here, the pH of all treatment solutions was within the range 7.6–7.8. This is an important result, suggesting that the compounds responsible for sperm activation in *C. intestinalis* and *A. aspersa* are biologically active in normal seawater and may therefore have adaptive significance.

Potential ecological implications

The results presented here suggest that the sperm of *Ciona intestinalis* and *Ascidiella aspersa* conserve energy resources until they are in proximity to eggs. This mechanism for extending sperm longevity may have important ecological implications.

The evolution of a mechanism that enables sperm to conserve energy reserves may enhance fertilization success by prolonging sperm viability until gametes are brought into contact through water currents. Such a mechanism is unlikely to be of any advantage if gametes are rapidly diluted by water turbulence and the probability of fertilization success declines sharply with time (Pennington, 1985; Denny and Shibata, 1989). Previous studies have shown that the probability of fertilization success declines quickly with increasing distance from a spawning individual. This decline is due to gamete dilution and occurs in environments of both high (Pennington, 1985; Denny and Shibata, 1989) and low energy (Yund, 1990). However, these experiments were conducted over restricted scales that are not representative of large numbers of individuals spawning over an extensive area. In such situations, gametes from any one individual may be very dilute at a short distance from the spawning animal, but the rate of sperm-egg collision may not be compromised if there is a high concentration of gametes from many individuals spawning over a wide area. This may be particularly true in sheltered low-energy environments.

Svane and Havenhand (1993) report that *C. intestinalis* occurs at densities of several thousand individuals per square meter over extensive areas of hard substratum in Swedish fjords. Similarly, we observed that *C. intestinalis* and *A. aspersa* completely dominated available substrate over an extensive area at the collection site. Both of these organisms are abundant in sheltered low-energy environments (Millar, 1953), and spawning in populations of ascidians appears to be synchronous in response to light

(Huus, 1939; Lambert and Brandt, 1967; Whittingham, 1967). Therefore, in contrast to the often cited examples of Pennington (1985) and Denny and Shibata (1989), we propose that the concentration of gametes from spawning *C. intestinalis* and *A. aspersa* would be high over an extensive area, and that this concentration could remain high for a considerable time due to the low energy of the environments in which these organisms live. Thus, a mechanism that extends the period of sperm longevity and hence fertilization success in these species may have considerable selective advantage.

In addition to enhancing fertilization success, a mechanism by which sperm conserve energy may have significant implications for the dispersal of these organisms. Because the adult stages of solitary ascidians are sessile, gene flow has been assumed to be primarily mediated by the planktonic egg, embryonic, and larval stages (*e.g.*, Havenhand, 1991). However, the eggs of both *C. intestinalis* and *A. aspersa* are surrounded by a viscous mucilage. Svane and Havenhand (1993) report that eggs of *C. intestinalis* are often spawned in this mucus, which readily adheres to available substrate, and that embryos and larvae of this species may be retained within strings of mucus until settlement occurs. Therefore, the egg, embryonic, and larval stages of *C. intestinalis* and *A. aspersa* may not mediate gene flow to the extent previously thought. If this is the case, then gene flow in these species may be significantly mediated by sperm, and the conservation of sperm energy may be important in facilitating this process.

These potential ecological implications of a mechanism by which sperm can conserve energy reserves and thereby extend the period of sperm viability are speculative. The adaptive significance of such a mechanism for fertilization success and sperm-mediated gene flow in free-spawning species will be placed in doubt if it is found to be shared by other members of the clade that do not form extensive populations at high densities in low-energy environments. Further, we emphasise that the results presented here were obtained under restricted conditions that do not reflect those in the natural environment of *C. intestinalis* and *A. aspersa.* Our procedure of incubating eggs in a small volume of water for 24 h produces an unknown concentration of unknown chemicals. Neither the existence nor the activity of these chemicals in the field has been shown. Thus, the results reported here may not be representative of the kinetics of sperm in the natural environment.

Given these limitations, the results reported here should be viewed as preliminary and treated accordingly. Further investigations are required to confirm the existence of a mechanism that enables sperm to prolong their viability by conserving energy reserves in the absence of eggs. Additionally, studies into the chemical composition and mode of operation of compounds that stimulate increased sperm activity in *Ciona intestinalis* and *Ascidiella aspersa* may yield substantial information on the mechanism of species specificity of both sperm activation and taxis.

Acknowledgments

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Structure of the Female Reproductive Tract in the Dungeness Crab (*Cancer magister*) and Implications for the Mating System

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Abstract. Anatomical and behavioral aspects of reproduction in female Dungeness crab, *Cancer magister,* were investigated. Female crabs were collected over a two-year period and external indicators of reproductive condition were recorded. A subset of crabs was retained for macroscopic and histological examination of the reproductive tract. In addition, males and females were held in the laboratory for mating observations, thus providing females of known mating history for dissection. The spermatheca is of the "ventral type," *i.e.,* the vagina and the oviduct open into the spermatheca in close proximity to each other. A novel organ that often contains sperm, the bursa, is separate and distinct from the spermatheca in both position and origin and is reported for the first time for a brachyuran crab. The bursa is located just inside the vulva, distal to the spermatheca, and opens into the vagina. The sperm plug of *C. magister,* described here for the first time, is unique in form among cancrids. The sperm plug is placed in the vagina by the first male to mate with a soft-shelled female and prevents subsequent access to the spermatheca. However, the sperm plug does not occlude the vulva nor prevent subsequent copulation; ejaculates from subsequent matings are deposited in the bursa. Hypotheses of the possible function of the bursa are discussed.

Introduction

Reproductive behavior of brachyuran crabs can be classified into two major patterns: (1) hard-shelled female mating, in which reproductive activity is not necessarily tied to the female's molt cycle (*e.g.,* some terrestrial species and "multiparous" majids), and (2) soft-shelled female mating (exemplified by portunids and cancrids) where copulation is only possible for a limited time after the female molts and before her shell hardens (Hartnoll, 1969; Salmon, 1983). In the latter pattern, courtship is prolonged and the typical sequence may include release of a pheromone (*e.g., Callinectes sapidus,* Gleeson, 1980 and 1982; Gleeson *et al.,* 1984) in combination with other sensory information (Dunham, 1988), "attendance" of the female by the male prior to the female's molt, molting followed by copulation, and post-copulatory guarding of the female (Salmon, 1983). Most brachyurans are polygynous, and Christy (1987) has characterized their mating associations based on the modes of competition among males for mates. Male cancrids (and portunids) exhibit "female centered competition," and search for and directly defend females several days before the female molts. In this type of mating system, post-copulatory guarding and sperm plugs are typical (Christy, 1987). Male cancrids tend to be polygynous (Cleaver, 1949; Butler, 1960; Snow and Neilsen, 1966; Orensanz and Gallucci, 1988) and in *Cancer magister* the sexes aggregate during a relatively short, synchronous breeding season (Orensanz and Gallucci, 1988).

After individuals have located and selected a mate and copulated, competition may continue at the level of male gametes. Sperm competition is defined as competition between the ejaculates, or sperm, of rival males for fertilization of a female's eggs (Parker, 1970). Little work on sperm competition has been done with brachyurans but their anatomy and behavior show analogies to those of vertebrates and other invertebrates where sperm competition has been demonstrated (Waage, 1979 and 1984; Diesel, 1990). In species with internal fertilization, three conditions must be met before sperm competition can occur: (1) the female must retain sperm, (2) the female

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must receive sperm from more than one male (either in a single molting/mating cycle or by storing sperm across cycles), and (3) the retained sperm must be used in fertilization (Parker, 1970). All female brachyurans have either ecto-mesodermally (spermatheca) or ectodermally derived (thelycum) sperm storage organs (Hartnoll, 1968). Diesel (1991) divided spermathecae into two morphological types: dorsal and ventral. The ventral type, occurring in the Calappidae, Geryonidae, Leucosiidae, Parthenopidae, Parathelphusidae, Corystidae, Ocypodidae, Majidae (see Diesel, 1991 for references), and Menippidae (Wilber, 1989), is a 'cul-de-sac' design with oviduct and vagina opening ventrally in close proximity to each other. The last male to inseminate a female should have the advantage (last male sperm precedence) because his sperm are placed nearest the oviduct, physically blocking access of rival sperm to the emerging oocytes. In dorsal-type spermathecae, the dorsally opening oviduct and the ventrally opening vagina are widely separated from each other, and the first male to inseminate the female is hypothesized to have the advantage (first male sperm precedence); oocytes encounter his sperm first. The dorsal type occurs in Portunidae and Pilumnidae and was predicted in the Cancridae (Diesel, 1991).

Sperm competition creates two opposing selective forces on males: (1) mechanisms for pre-emption (replacement or displacement) of stored rival male sperm, and (2) adaptations that resist pre-emption by rival males (Parker, 1970). The gonopods of the snow crab, *Chionoecetes opilio,* are equipped with backward pointing processes and it is possible that they are used to remove sperm previously placed in the female's spermathecae (Beninger *et al.,* 1991); such a mechanism has been demonstrated in damselflies (Waage, 1979). Brachyuran sperm plugs may prevent subsequent intromission (Parker, 1970; Diesel, 1990); alternative hypotheses about their function include protection or retention of sperm (Spalding, 1942; Ryan, 1967; Hartnoll, 1969) and nourishment of sperm during storage (Cronin, 1947; Adiyodi and Anilkumar, 1988).

Reproductive dynamics of *C. magister,* including behavior and the structure and function of the reproductive organs, are poorly understood. Females reach sexual maturity at the first adult instar, at approximately 100 mm carapace width (Weymouth and MacKay, 1936; Orensanz and Gallucci, 1988), but at least some females mate when molting from the final juvenile instar to the first adult instar (Cleaver, 1949; Butler, 1961; Poole, 1967; Hankin *et al.,* 1985; Orensanz and Gallucci, 1988). Females retain sperm across molts (Shirley and McNutt, 1989) and 'additional' males add to the spermathecal sperm supply during annual molting and copulation. Some adult females 'skip-molt' (Ebert *et al.,* 1983) and a tagging study has shown that such females can produce viable egg clutches from stored sperm for at least 2.5 years (Hankin *et al.,* 1989). As mentioned above, sperm plugs are typically found in post-copulatory female cancrids (Hartnoll, 1969) but have not been described for *C. magister,* although they are prominent in *C. pagurus* (Edwards, 1966), *C. borealis* (Elner *et al.,* 1985), *C. irrotatus* (Elner and Stasko, 1978), *C. gracilis* (Orensanz *et al.,* 1995), and five other species of *Cancer* from the Pacific coast (pers. obs.).

In this report, we describe a new structure of the female reproductive tract of *C. magister* and discuss its possible functions. We also describe and discuss the sperm plug, the morphology of the spermatheca, and mating behavior in the context of hypotheses about the mating system.

Materials and Methods

Collections, histology, and scanning electron microscopy

Female crabs (76.3–169.0 mm carapace width) were collected by SCUBA or crab pots from Ship Harbor or Mukilteo, Washington (Fig. 1) at approximately one-month intervals, more frequently during the mating season (generally May–July in Puget Sound), from 4/90 through 6/92. Carapace width (CW), excluding the tenth antero-lateral spines, and shell condition (SC) (Table I) were recorded for each crab as were external indicators of reproductive activity such as the presence or absence of sperm plugs, eggs, or egg cases. Crabs were either released or retained for laboratory observation, experimen-

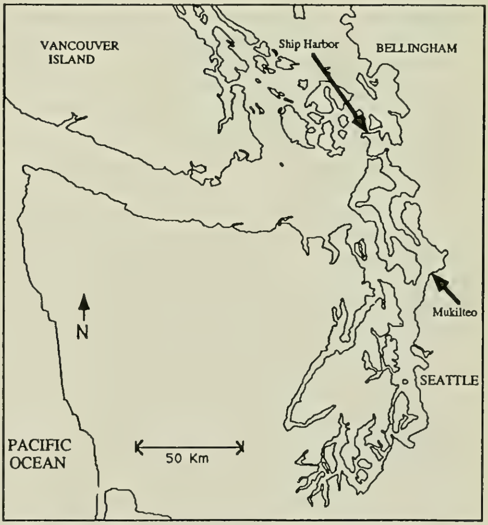


Figure 1. Location of collection sites for *Cancer magister* in Washington State, USA.

Table I

Definition of shell condition (SC) categories in Cancer magister

SC	Time since last molt	Description
0	0–24 hours	Entire exoskeleton pliable, carapace not hardened; setae on carapace and pleopods orange.
1	*ca.* 1 mo.	Underside of carapace and merus of legs pliable. Shell white ventrally; setae on shell and pleopods may be orange, cream or brown.
2	*ca.* 1–3 mos.	Shell not pliable, *i.e.*, hardened, cream to golden ventrally; setae on pleopods brown.
3	*ca.* 3–12 mos.	Shell hardened, golden ventrally. May be epibiotic growth (usually barnacles). May be some darkened areas on shell (scars or chitinoclastic bacteria).
4	+12 mos.	Shell orange ventrally. May be heavy epibiotic growth, especially barnacles. Usually blackened areas on shell (scars, bacterial growth, etc.)

tation, or dissection. Dissections were performed over ice, and the reproductive tracts were removed and the ovary severed from the rest of the reproductive organs 1–2 cm from the oviduct. One side of the lower genitalia was dissected and examined under a dissecting microscope. The remaining side of the lower genitalia was placed in Bouin's fixative for 24 h, transferred to 50% ethanol for 24 h, and then placed in 70% ethanol.

Tissue samples fixed in Bouin's were cleared in xylene, embedded in paraffin, sectioned at 3–4 μm on different planes depending on the structure of interest, and stained with standard hematoxylin and eosin (H & E) for general tissue differentiation. The presence of chitin in the reproductive tract was demonstrated by Chatton's stain (Grey, 1975) and melanin in the spermatheca by the acid silver nitrate argentaffin reaction (Sheehan and Hrapchak, 1980). To determine whether bacteria were present and to compare the condition of the bursal and spermathecal contents, paired sections of bursa (described in Results) and spermatheca from a female nearing extrusion (SC3, CW = 133.9 mm) were stained with Taylor's Brown-Brenn modified Gram's, Giemsa, Ziehl-Neelsen's acid-fast, and periodic acid-Schiff (PAS) stains (Sheehan and Hrapchak, 1980). To ensure correct evaluation of stained sections, appropriate control slides were simultaneously stained with crab tissue sections.

For scanning electron microscopic (SEM) observations, sperm plugs were dehydrated in an ascending ethanol series, sublimated in Peldri II® (Ted Pella, Inc., Redding, CA) and sputter coated with 300 Å gold palladium. The sperm plugs are hard objects and required no special histological fixatives before processing for SEM. Observations were made on a JEOL 840A microscope.

Female molt casts were collected opportunistically from the beach and by SCUBA from Mukilteo from April to October 1992 and the presence and condition of shed bursae (see Results) recorded.

Behavioral observations and manipulations

Behavioral observations were conducted, variously, at Shannon Point Marine Center, Anacortes, the Mukilteo National Marine Fisheries Service (NMFS) Field Station, and Friday Harbor Laboratories, San Juan Island, Washington. For observations of mating, paired crabs collected in the field and pairs that formed in the laboratory were used. Unpaired males and females were held communally in tanks of various sizes and females were considered to be nearing ecdysis when embraced by a male. Pairs were placed in flowing seawater in either 38-l aquaria with gravel or sand substrate or 1.8 m diameter × 0.3 m deep wading pools with sand substrate.

To investigate the time period during which a female is receptive to copulation and the possibility of multiple matings, different males were introduced at various intervals for up to 16 days after females (n = 11) had molted, the crabs were observed until they had either copulated or until both were nonreactive to the presence of the other, and then the male was removed. Once a female became non-receptive, she was dissected, and the condition of the spermathecae and bursae and presence of sperm plugs were noted. The genitalia were then fixed in Bouin's. After fixation, the volume of material (spermatophores and seminal fluid; see Results) in each bursa was determined by measuring displacement of a known volume with a digital micropipet. Six additional females held in the laboratory were allowed to molt and mate only once for comparison of bursal content volumes to the bursal content volumes of the multiply mated females.

To investigate the origin of sperm plugs, 40 pre-ecdysial females were held separately in 38-l aquaria with sand substrate and flowing seawater, either with (n = 20) or without (n = 20) a male. Females were sacrificed and dissected at approximately 24-hour intervals post-molt for 20 days. The lower genitalia were removed and examined under a dissecting microscope and then fixed in Bouin's.

Results

Ovary and oviduct

The bilobed ovary extends from behind the tenth antero-lateral carapace spines along the anterior edge of the carapace medially to the stomach, then posteriorly along the stomach with the two halves joining behind the stomach by a commissure or isthmus. The ovary then dips ventrally under the pericardial sac and runs asymmetrically along the interior of the shell to the last thoracic segment on the left and just into the second abdominal somite on the right. On both sides there is a short lateral extension at the level of the second or third walking legs; the ovary is held in place by connective tissue. The ovary is joined to the dorsal surface of the spermatheca at the level of the second walking leg via a short oviduct that is normally hidden from view by the ovary. The oviduct enters the spermatheca near the opening to the vagina (Fig. 2) and is composed of a layer of columnar epithelium with a thin chitin lining. No valve or similar structure is present at the junction of the oviduct and spermatheca.

Spermatheca (seminal receptacle)

The spermatheca (Fig. 3) is a flattened pouch held firmly in place by connective tissue to the posterior wall of the sternal muscle chamber of the second walking leg. The spermatheca is thin walled, not distensible, fragile, and approximately 10 mm long by 7 mm wide (all anatomical measurements given are averages from 5 females with CWs of ~ 135 mm). The ventral wall and a small portion of the dorsal wall are composed of a layer of chitin-lined columnar epithelial cells resting on a basement membrane and bounded on the exterior by connective tissue. Muscle elements are found within the connective tissue but they do not appear to insert onto the walls of the spermatheca. For most of the dorsal wall, the columnar epithelium and chitin lining are replaced by a specialized secretory epithelium, referred to hereafter as the dorsal epithelium. Before and for a relatively short time after molting, the dorsal epithelium proliferates and 'mushrooms' into the spermathecal lumen overlapping the neighboring chitin-lined columnar epithelium (Fig. 4). At any time of the year, but especially when the dorsal epithelium is enlarged, granules of melanin are found near and over the surface of the dorsal epithelium. A conspicuous brown to black granular area composed of melanin, often forming concentric rings or bands, is almost invariably present in the lumen in the blind end of the spermatheca, occasionally reaching but never extending into the vagina (Fig. 3). The bands tend to be dark in large females and faint in smaller females and are present in virgin females (< 100 mm CW and without sperm in the spermathecae). If present, spermatophores or sperm are free in the lumen and little or no seminal fluid is present. Unless the spermatheca is full, sperm are usually concentrated near the vagina and oviduct. Because the spermatheca is translucent when empty, it is difficult to distinguish it from surrounding connective tissue unless the melanin is visible; stored sperm give the spermatheca a white appearance.

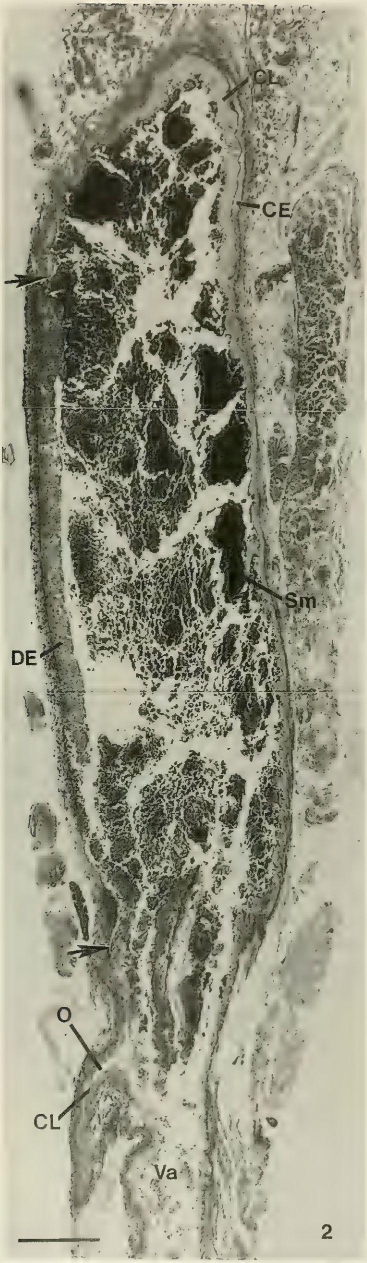
Examination of exuvia and histological sections of genitalia from recently molted females revealed that the entire chitin lining of the reproductive tract is shed at molt but the old lining of the vagina breaks at or near its junction with the spermatheca so that the shed lining of the spermatheca is retained rather than lost with the exuvium. In post-molt, unmated females, the sloughed chitin lining of the spermatheca is separated from the new lining but remains in place. In post-molt, recently mated females, this lining is found at the blind end of the sac (presumably displaced by the new ejaculate) and eventually degrades. The degrading chitin and melanin bands are usually found in association with each other.

Bursa, vagina (gonoduct), and vulva (gonopore)

The vagina is of the simple type (*sensu* Hartnoll, 1968), ~ 2 cm long from spermatheca to vulva and its layers of columnar epithelium, chitin, and connective tissue are continuous with those of the spermatheca. Attached to the internal surface of the vulva, a layer of longitudinal muscle forms a sheath that encloses the vagina, thinning and disappearing as it approaches the spermatheca. For at least a few days before and two weeks after molting, the muscular sheath is firmly attached to the sternite; at other times of the year it is less firmly attached and easily detached from the sternite during dissections.

Located between the vulva and the spermatheca and opening broadly into the medial side of the vagina is a translucent white or yellowish sac, the bursa (from the Latin *bursa* for sac) (Figs. 3, 5). Layers of the bursa (*i.e.*, connective tissue, epithelium, and chitin; Fig. 6) are continuous with those of the vagina, but the muscular sheath does not extend over the bursa. The sac is embedded in the muscles attached to the sternite, as is the vagina, but no muscles attach to the bursa. There is no histological evidence of glandular or secretory activity. Bursae often contained free sperm or a firm white substance consisting of seminal fluid and embedded spermatophores. The bursa is crumpled when empty and upon insemination ejaculate increases the volume of the sac, but the walls are not distensible. Fully expanded, the bursa is approximately 5 to 6 mm in diameter and has a maximum capacity of ~ 100 μl. For females of known mating history (*i.e.*, those that molted and mated in the laboratory) and those collected in the field, sperm were never found in the bursae unless there were also sperm in the spermathecae; however, sperm were not always present in the bursae, even if there were sperm in the spermathecae ($n = 184$ females dissected: 54 females with sperm in the spermathecae only, 119 females with sperm in the spermathecae and bursae, 0 females with sperm in the bursae but not the spermathecae, 11 females with both the spermathecae and bursae empty).

The vaginal lumen tapers from ~ 4 mm wide at the vulva to 0.5 mm just proximal (unless otherwise noted, the terms proximal and distal will be used with respect to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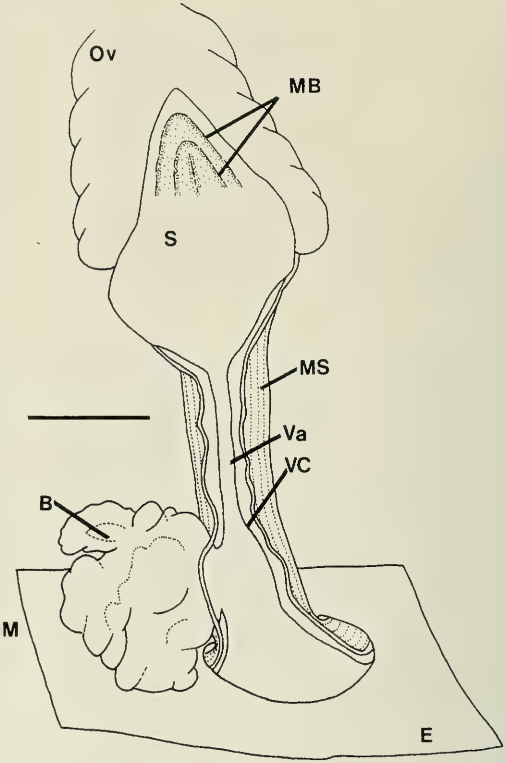
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the ovary) to the bursa, at an area referred to as the vaginal constriction (Fig. 3), and remains this width until joining the spermatheca. Within the narrow portion of the vagina, usually near the vaginal-spermathecal junction, the sloughed chitin lining breaks during exuviation, resulting in the lining of the bursa and its contents, and at least the distal portion of the vagina, being shed with the exuvium (Fig. 7). Depending on the location of the break, the proximal portion of the vaginal lining may remain in the vagina continuous with the lining of the spermatheca. The opening into the bursa is wide, ~ 2.5 mm, and seldom breaks, but occasionally the old bursal lining is retained.

In laboratory aquaria, the thin chitin linings of the genitalia disappear quickly (~ 1 week) from molt casts; however, of 90 field-collected female exuvia (CWs ≥ 100 mm) with intact sterna, 79 retained the lining of at least one bursa. Of those 79 exuvia, sperm were present in the bursae in 20 (25%).

The vulva, which is located on the sternite of the sixth thoracic segment slightly distal (with respect to the midline of the body) and anterior to the center of the sternite, is oval and has a conspicuous scale on its anterior edge (Fig. 8). Just inside the vulva, the vaginal lumen is collapsed and the gonopore is normally in the form of a closed slit. Nemertean worms are occasionally present just inside the vulvae and near the openings to the bursae.

Spermathecal and bursal contents

In females that have recently mated for the first time, the material in the lumen of the spermatheca is white and consists of free spermatophores. Spermatophores adhere to each other but never form a solid mass, nor are they embedded in any sort of material; little or no seminal fluid is present. Spermatophore walls are eosinophilic and the sperm within are basophilic. By the time of egg extrusion, several months after ecdysis and copulation, the spermatophore walls have disappeared but the consistency and gross appearance of the material remains the same. In recently mated females that have stored sperm (spermatophore-free) from a previous mating(s), newly deposited spermatophores are nearest the vagina and oviduct with the stored sperm immediately behind. The newly deposited and stored sperm are indistinguishable by consistency and gross (macro) appearance alone. Once the spermatophore walls have disappeared, 'new' and 'old' sperm within the spermatheca cannot be distinguished visually or by differential staining; spermathecal sperm tend to stain evenly and consistently and are strongly basophilic, fuchsinophilic, and PAS positive.

Material freshly deposited in the bursa consists of spermatophores embedded in a solid white to yellow waxy substance. Spermatophores and the sperm within appear identical to those in the spermatheca in PAS and H & E staining affinities and overall appearance, except that they are embedded in an amorphous eosinophilic matrix, presumably seminal fluid. By the time of egg extrusion, appearance of the bursal contents has changed. While some of the sperm are still within spermatophores and embedded in seminal fluid, the mass has usually softened and there are frequently free sperm in the lumen. Often the softened mass is discolored and has a faint green or brown hue. Histological appearance of free sperm differs markedly from that of the spermathecal sperm. Many bursal sperm are misshapen, swollen, shriveled, or fragmented. Individual bursal sperms tend to stain unevenly and inconsistently, and the affinity for the stains differs from that exhibited by spermathecal sperms with many bursal sperms staining either more or less intensely and some not staining at all. After egg extrusion, a few to several oocytes frequently remain in the spermathecae but none are found in the bursae. No bacteria were detected in either the bursa or the spermatheca by acid-fast or Gram's stains.

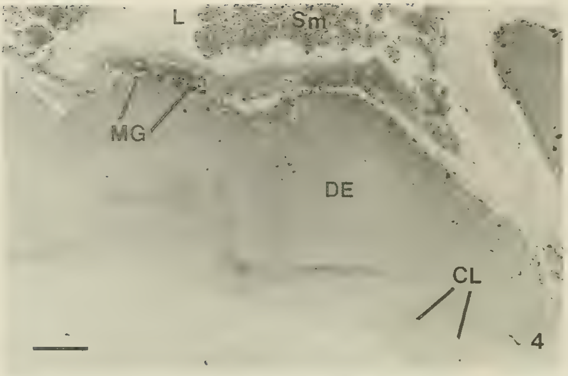
No differences in gross anatomy were observed between size classes of multiparous females or between multiparous and primiparous females. Females assumed to be virgin by virtue of their carapace width (< 100 mm) differed in that their spermathecae and bursae were always empty and their ovaries often indistinguishable from surrounding tissues.

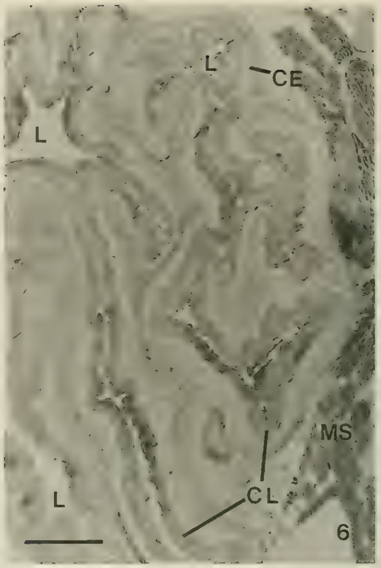
Sperm plugs

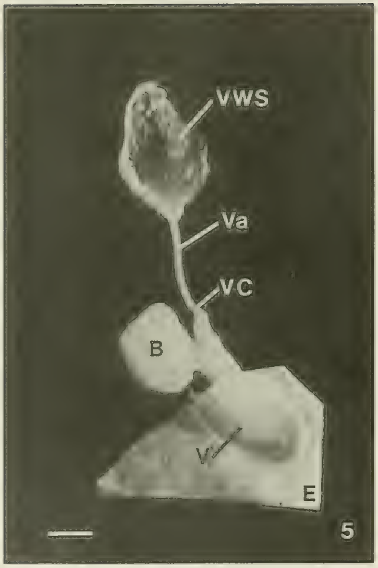
All females dissected after copulation had sperm plugs reaching from just inside the spermatheca to the bursa or just into the bursa, and frequently extending just outside the vulva (Figs. 8, 9). The sperm plus is composed of two distinct sections. The innermost, or proximal, section is ~ 10 mm long and extends from within the spermatheca

Figure 2. H & E. Longitudinal section of the spermatheca from a SC3 female (CW = 123.4 mm). Note the relative position of the oviduct to the vagina. Scale bar = 0.4 mm. CE, columnar epithelium; CL, chitin lining; DE, dorsal epithelium; O, oviduct; Sm, sperm; Va, vagina; arrows indicate junction of dorsal and columnar epithelium.

Figure 3. Camera lucida drawing of the lower genitalia of a female *Cancer magister* (SC2, CW = 152.0 mm). The muscular sheath has been cut open to reveal the vagina. Scale bar = 5.0 mm. A, anterior; B, bursa; E, inner surface of sternite; M, medial; MB, melanin bands; MS, muscular sheath; Ov, ovary; S, ventral surface of spermatheca; Va, vagina; VC vaginal constriction.







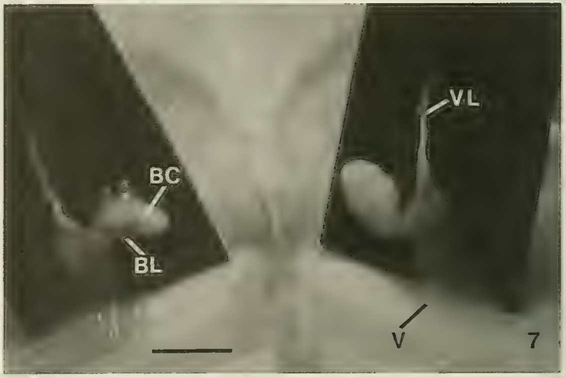
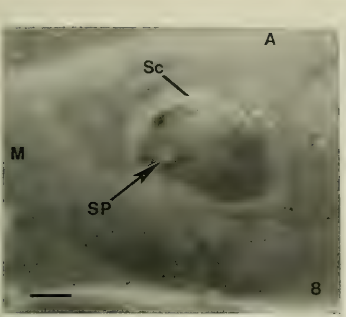


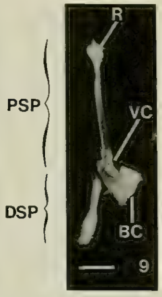
Figure 4. H & E. Longitudinal section of the spermatheca from a SC0 female (CW = 126 mm). Scale bar = 25 μm. CL chitin lining; DE, dorsal epithelium; L, lumen; MG, melanin granules; Sm, sperm.

Figure 5. Lower genitalia of a SC1 female (CW = 115.2 mm). All soft tissue, including the dorsal wall of the spermatheca, has been removed leaving only the chitin linings and the sternite. Scale bar = 2.5 mm. B, bursa; E, exterior surface of the sternite; V, vulva; Va, vagina; VC, vaginal constriction; VWS, ventral wall of spermatheca.

Figure 6. Chatton's stain for chitin. Longitudinal section of empty bursa from a SC1 female (CW = 128.8 mm). Opening to vagina is not shown. Note the maze-like appearance of the lumen of the empty, crumpled sac. Scale bar = 0.5 mm. CE, columnar epithelium; CL, chitin lining; L, lumen; MS, muscular sheath.

Figure 7. Shed bursal linings and contents (sperm) in exuvium (SC3; CW = 98.5 mm). Scale bar = 3.0 mm. BC, bursal contents; BL, bursal lining; V, inner surface of vulva; VL, vaginal lining.





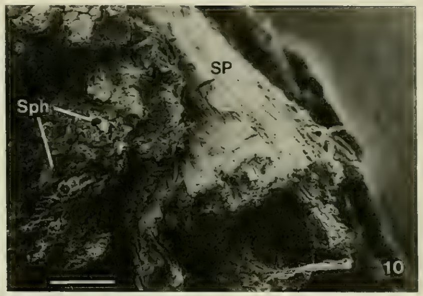


Figure 8. Right vulva with tip of sperm plug visible of a recently mated SC1 female. Scale bar = 2.0 mm. A, anterior; M, medial; Sc, scale; SP, tip of sperm plug.

Figure 9. Sperm plug and bursal contents of a female *Cancer magister.* The sperm plug has been removed from the female's reproductive tract. Scale bar = 2.5 mm. BS, bursal contents; DSP, distal sperm plug (head of which protrudes from vulva); PSP, proximal sperm plug; R, "root" of proximal sperm plug (enters spermatheca); VC, area of vaginal constriction.

Figure 10. Scanning electron micrograph of the root end of the proximal sperm plug. Note its fibrous nature. Scale bar = 100 μm. Sph, broken spermatophores; SP, sperm plug.

to just beyond the vaginal constriction and sometimes to the bursal entrance, occluding the proximal portion of the vagina but never sealing off the bursa. The innermost section of sperm plug is translucent white, firm but slightly pliable, and cannot be separated from the vaginal wall without destroying the vagina. The end entering the spermatheca has a root-like appearance while the other end is club-shaped or, if extending into the bursa, forms into a y-shape with short blunt arms. In histological sections, the proximal section of plug is eosinophilic, does not stain with Chatton's stain, and appears amorphous; embedded spermatophores are seldom found. SEM examination revealed that the plug is composed of numerous tightly packed lengthwise fibers (Fig. 10).

The distal, or outermost, section of the sperm plug extends from the end of the proximal plug to slightly outside the vulva. The opaque white distal section is shorter than the proximal section but about the same width and, therefore, does not fill the vaginal lumen or occlude the vulva (Fig. 8). The distal plug does not adhere to the vaginal wall, is easily broken and, in captive specimens, the visible part is usually lost within hours to days of copulation; protruding plugs are rarely seen in females in the field. Out of 4 SC0, 55 SC1, and 111 SC2 (Table I) females collected in the field (excluding those SC2 females with extruded eggs), only 1, 3, and 6 females, respectively, had visible sperm plugs protruding from their vulvae. Of the females that did not have plugs visible in their vulvae, 2 SC0, 8 SC1, and 20 SC2 were dissected, and 1, 3, and 2, respectively, had part of the distal plug within at least one vagina; all had the proximal portion of the plug. The distal section is never found in SC3 females and neither section is ever found in SC4 females, or those carrying eggs or empty egg cases. The distal plug section is similar in H & E staining affinity and appearance (SEM) to the proximal portion.

Sperm plugs are not found in unmated females although sections of the proximal vagina are frequently occluded with unconsolidated matter and, unlike the sperm plugs found in mated females, the occluding material can be removed with forceps without damaging the vagina.

Multiple mating

Dissections revealed that all six singly mated females and all 11 multiply mated females had proximal sperm plugs between the bursa and spermatheca. The presence of sperm plugs does not prevent subsequent copulation by males of mated females. Behavior at copulation for multiply mated females appears no different than that of previously unmated females. Females are receptive or attractive to males and will copulate for up to 10 days postmolt (Table II). With subsequent matings, there is an increase in the volume of material in the bursae ($P \ll 0.01$; $r^2 = 0.576$), until maximum capacity is reached (Fig. 11). Multiple copulations do not result in a concomitant increase in spermathecal content. The bursae of three females which copulated 4, 9, and 4 times were full. No material was found in the bursae of the six singly mated females. Additional mating pairs held in the laboratory and allowed to remain together through the post-copulatory guarding period (of approximately three days) often copulate more than once during the guarding period. Of 124 females of ≥ 100 mm CW collected from Ship Harbor and dissected, all had sperm in the spermathecae and of these 94 (76.4%) also had sperm in the bursae. Of these 94 females, 14 had bursae 75 to 100% full while 21 had bursae 50 to 75% full.

Observations of mating indicate that no more than 50% of the length of the first male gonopod enters the vagina. Manipulations of an excised male (CW = 150.1 mm) first gonopod and female (CW = 135.0 mm) genitalia reveal that the male gonopods are too wide to penetrate beyond the constriction of the vagina that occurs just interior to the bursa and therefore cannot reach the spermatheca (Fig. 12).

Upon removal of males from females' tanks immediately after termination of copulation, it was often noted that a small firm white mass was clinging to the tips of the first gonopods of the males. Examination of wet mounts of this material under a compound microscope revealed many fibers and few spermatophores.

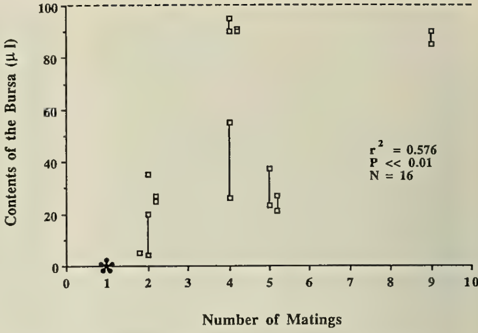


Figure 11. Relationship between the number of matings and the volume of material (spermatophores and seminal fluid) in the bursa. Linked values (☐ — ☐) represent the volumes from the left and right bursae of a single female; averaged volume of left and right bursae for each female was used in calculations. The * represents the six singly mated females. Bursal capacity is ~ 100 μl.

Discussion

The reproductive anatomy of female *C. magister*, with the exception of the bursa, resembles that reported for other brachyurans (Cano, 1891; Pearson, 1908; George, 1963; Ryan, 1967; Hartnoll, 1968; Johnson, 1980; Beninger *et al.*, 1988; Bawab and El-Sherief, 1989; Diesel, 1989) being bilaterally symmetrical with paired vulvae, vaginae, spermathecae, and oviducts leading to the branched ovary. Below we discuss particular features of the spermatheca, sperm plugs, and bursa.

Spermatheca

No valve-like tissue was observed at the spermatheca-oviduct junction as was found in the grapsids *Eriocheir sinensis, E. japonicus* and *Hemigrapsus sanguineus* (Lee and Yamazaki, 1990).

The ventral position of the oviduct, in close proximity to the vagina, does not support Diesel's (1991) suggestion of a dorsal-type spermatheca in cancrids. Furthermore, even though the spermatheca is of the ventral type, last male sperm precedence may not be in operation. In Dungeness crabs sperm competition would be between sperm stored from mating events of previous years and newly deposited sperm from the first copulation after the female's most recent molt. Because the sperm plug blocks access to the spermatheca, sperm from extra copulations

Table II

Period of female 'receptivity/attractiveness' and volume of bursal contents

Female	Post-molt CW (mm)	Day 0	1	2	3	4	5	6	7	8	9	10	11–15	16	No. copulations	Bursal content (μl) Left	Right
A	138.4	C	CC												3	*	*
B	161.0	C	C	—	—	N	N								2	20	4
C	162.9	C	—	—	C	—	—	N							2	27	25
D	146.2	C	C	C	C	—	N	—	—	N	N				4	55	26
E	167.5	C	—	—	—	—	—	—	C	N					2	*	35
F	146.1	C	—	—	—	C	C	—	C						4	91	90
G	140.7	C	C	—	C	—	—	C	C						5	21	27
H	134.7	C	—	—	—	—	—	—	—	C	—	N			2	5	5
I	154.8	C	C	C	C	C	C	C	C	C	N	N			9	85	90
J	148.4	C	?	C	C	—	—	—	—	—	C				4	90	95
K	161.2	C	—	—	—	—	C	NNN	C	—	C	C	—	N	5	23	37

Males introduced were as large as or larger than the soft-shelled female. Day 0 is day of female's molt. Female A, Day 1: two different males introduced separately and copulated. Female K, Day 6: 3 males introduced, no copulations. C = copulation occurred; N = no copulation occurred; — = no trial; * = not measured.

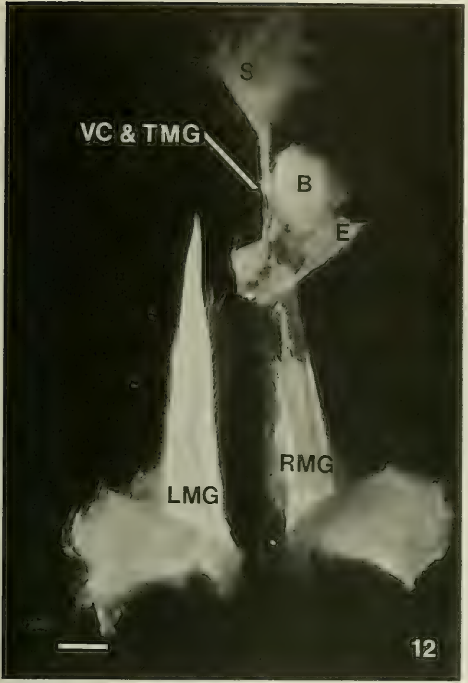


Figure 12. Excised male (CW = 150.1 mm) and female (CW = 135.0 mm) genitalia. The excised right first gonopod has been pushed into excised right genitalia of a female; note that the gonopod cannot penetrate beyond the vaginal constriction. The end of the right gonopod has been colored with a black marker. The left first gonopod is shown for reference. Scale bar = 5.0 mm. B, bursa; E, inner surface of sternite; LMG, left male gonopod; RMG, right male gonopod; S, spermatheca; TMG, tip of male gonopod; VC, area of vaginal constriction.

enter the bursa and presumably are not involved in fertilization (see discussion of bursa below). Unlike the majids which Diesel used to develop his model, *C. magister* does not utilize "sperm gel" (*sensu* Diesel, 1991) to segregate rival male sperm contributions within the spermatheca, nor can the male gonopods reach into the spermatheca to remove stored sperm as may occur in *Chionoecetes opilio* (Beninger *et al.,* 1991). Even though the addition of new spermatophores displaces previously stored sperm towards the blind end of the spermatheca, without some mechanism to maintain their initial segregation there is the potential for mixing of new and old sperm in the lumen of the spermatheca. The spermatheca rests against the sternal chamber muscles of the second walking leg and some degree of mixing caused by locomotor activities between the time of mating and egg extrusion four to six months later is conceivable. Mixing may also occur during extrusion of the oocytes through the spermatheca. Depending on the degree of mixing, there may be no sperm precedence.

Most authors report that the dorsal epithelium of the spermatheca is secretory, but the function of the secretions is unknown. It has been suggested that they are involved in sperm plug dissolution (Spalding, 1942), sperm plug formation (Bawab and El-Sherief, 1989), sperm dehiscence (Ryan, 1967; Adiyodi and Anilkumar, 1988; Diesel, 1989), maintenance of the sperm (Johnson, 1980), and maintenance of a bacterial population within the spermatheca (Elner and Beninger, 1992; Beninger *et al.,* 1993). In *C. magister,* the dorsal epithelium is probably responsible for the melanin present in the lumen of the spermatheca. Melanin has two primary functions, protective and destructive, and is involved in immune responses to the presence of foreign substances and diseased or dead host tissue in a number of groups, including crustaceans (Salt, 1970; Wolke *et al.,* 1985; Soderhall and Smith, 1986). Melanin is bacteriocidic (Wolke *et al.,* 1985) and the large amount present in the spermatheca may be responsible for excluding bacteria and other harmful agents from the spermatheca. Within *C. opilio* spermathecae, the epithelial secretions are hypothesized to provide a favorable culture medium for a morphologically homogeneous population of bacteria which through their end products may either exclude opportunistic microbes from the spermatheca or provide substrates for the sperm to metabolize (Elner and Beninger, 1992; Beninger *et al.,* 1993). In contrast to *C. opilio,* no bacteria were found in the spermathecae of *C. magister*; the presence of melanin may exclude bacteria from the spermatheca. In addition, melanin protects cells by absorbing free radicals produced during metabolism, and in cryophilic fish has been shown to absorb free radicals formed during lipid oxidation (Wolke *et al.,* 1985). Sperm stored in the spermatheca of *Scylla serrata* (Portunidae) maintain a low level of anaerobic metabolism (Jeyalectumie and Subramoniam, 1991); in *C. magister* melanin may promote sperm storage by absorbing free radicals produced during metabolism.

Sperm plugs

The sperm plugs are atypical in that they do not occlude the vulva and obstruct only half the length of the vagina, contrary to what has been indicated for several congeners, including *C. irroratus* (Chidester, 1911), *C. pagurus* (Edwards, 1966), *C. borealis* (Elner *et al.,* 1985), *C. gracilis* (Orensanz *et al.,* 1995), and five other Pacific coast species of *Cancer: C. antennarius, C. anthonyi, C. branneri, C. oregonensis,* and *C. productus* (pers. obs.).

Two types of structures are referred to in the literature as sperm plugs and they may have different functions

(Diesel, 1991). The 'internal' type of sperm plugs seen in portunids (Spalding, 1942; Hartnoll, 1969) and some majids (Diesel, 1991), does not block access to the spermatheca or prevent subsequent matings, but seals off or segregates rival sperm masses *within* the spermatheca. The second or 'external' type, typically found in cheiragonids (Armetta and Stevens, 1987), menippids (Tomikawa and Watanabe, 1990), and cancrids (Orensanz *et al.,* 1995) fills the vagina and blocks access to the vulva thereby preventing subsequent copulations. Although they do not occlude the vulva, the sperm plugs of *C. magister* fill the proximal portion of the vagina blocking access to the spermatheca and appear to be derived from the external type of plug. Because seminal fluid is found in the bursa, but not the spermatheca, it seems likely that the male terminates copulation with seminal fluid which becomes the sperm plug in the case of the female's first copulation following ecdysis, but in the case of subsequent copulations, remains in the bursa to form the matrix typically associated with the spermatophores. The fibrous nature of the material adhering to the tip of the male gonopods after copulation supports this conclusion.

Diesel (1991) suggested that females are responsible for producing the external plugs by forcing material (seminal fluid) from the spermatheca into the vagina after copulation, and that this plug type is in the interest of the female because it prevents further copulation and reduces male-male competition while the female is soft-shelled and vulnerable to injury. In *C. magister* it seems improbable that contractions of the spermatheca could achieve the high degree of organization of the fibers that make up the plug because the spermatheca itself is not muscular and the limited number of muscle elements associated with its connective tissue seem insufficient for the task, particularly during the post-molt stage, while the exoskeleton is still soft. Furthermore, manipulations of excised female genitalia and male intromittent organs revealed that the gonopods cannot penetrate beyond the point of the vaginal constriction, which is approximately where the proximal section of the sperm plug begins; male *C. magister* may be unable to determine if a soft female is already inseminated. Because males probably cannot detect plugs, the plugs cannot reduce male-male competition for copulation, but instead are placed by the first male to prevent subsequent male contributions to the spermatheca.

Bursa

A sperm plug is formed at the end of the first copulation following molt; however, it is effective in blocking the vagina only between the spermatheca and the bursa. This plug prevents further male contributions to the spermatheca but does not preclude additional mating; ejaculates from subsequent copulations enter the bursa.

As spermatophores do not enter the bursa until a second copulation, the presence of sperm in the bursa (or in the shed lining in the exuvium) is a conservative indicator of whether a female mated during her previous molt. Ninety-four (76.4%) of 124 sexually mature mated females (sperm in spermathecae) collected from Ship Harbor had sperm or spermatophores in their bursae indicating that a second copulation may be common; however, it was not possible to determine whether these females were copulated twice by the first male to copulate, as often occurs during post-copulatory guarding in the laboratory, or a second male. Examination of cast exuvia is non-invasive and may be useful as a comparative index of mating activity of a particular locale from year to year.

The difference in appearance of male product in the spermatheca versus the bursa is noteworthy. When one vulva of a receptive female was blocked with duct tape and a male was allowed to copulate with her, the ejaculate released into the tank by the male crab formed an amorphous mass that was indistinguishable in color, texture, and consistency from the material normally found in the bursa (pers. obs.) suggesting that seawater enters the bursa during or shortly after intromission. The sperm in the spermatheca never formed a hardened mass, suggesting either that seawater does not enter the spermatheca during intromission or that a hardened mass is not formed because there is no seminal fluid to interact with the seawater (after intromission the sperm plug would prevent entry of seawater into the spermatheca). Because stored sperm are used to produce viable egg clutches (Hankin *et al.,* 1989), if any harmful waterborne agents typically enter the spermatheca during intromission, they must be effectively 'neutralized' by the storage conditions within the spermatheca; the environment within the bursa is probably not comparable to that within the spermatheca.

We offer four possible hypotheses to explain the function of the bursa:

(1) *Short-term sperm storage.* Even though the shedding of the lining of the bursa and its contents at ecdysis precludes a role in long-term sperm storage, the bursa could function in short-term sperm storage. However, this seems unlikely for several reasons. Although the bursa can hold a large volume of sperm and receives sperm after the spermatheca is sealed off by the proximal section of the sperm plug, the sperm do not appear to be well protected from the outside environment. Seawater probably enters the bursa during or shortly after intromission and likely introduces harmful agents. Additionally, nemertean worms were often seen near the opening to the bursa and material within the bursa was frequently discolored. At egg extrusion the bursal sperm were either still within spermatophores and embedded in seminal fluid or free in the bursal lumen but in a degenerative state. There is

no obvious path for oocytes to travel through the bursa during extrusion nor does it seem reasonable that movements of the second walking leg muscles could somehow direct sperm from the usually crumpled and convoluted bursa into the vagina to meet the oocytes. After egg extrusion residual oocytes were frequently found in the spermatheca; however, none were ever found in the bursa.

(2) *Secretory function.* Although the bursa is well situated to be involved in egg attachment or some other activity related to reproduction, there is no histological indication of secretory ability.

(3) *Relict structure.* Primitive brachyurans with sperm storage organs that open externally without any connection to the oviducts (thelyca) lose stored sperm at ecdysis (Hartnoll, 1975). The bursa of *C. magister* could represent a type of thelycum that has lost its function as the spermatheca evolved. The spermatheca is an enlargement of the genital duct with a non-chitinized portion of mesodermal origin and a chitinized portion of ectodermal origin (Payen, 1974), while thelyca are invaginations of the sternal integument (Hartnoll, 1975).

If the bursa is an archaic structure one might expect to find it in other members of the genus or in some outgroup. Nations (1979) subdivided the genus *Cancer* into four subgenera: *Romaleon, Metacarcinus, Cancer sensu stricto,* and *Glebocarcinus. C. magister* was placed in *Metacarcinus,* which was concluded to be intermediate between *Romaleon,* the more primitive group, and *Cancer sensu stricto,* the more advanced group. Dissections of mature female *C.* (*Metacarcinus*) *anthonyi, C.* (*M.*) *gracilis, C.* (*M.*) *borealis, C.* (*Romaleon*) *branneri, C.* (*R.*) *antennarius, C.* (*R.*) *jordani, C.* (*C. irroratus, C.* (*C.*) *productus,* and *C.* (*Glebocarcinus*) *oregonesis* revealed no evidence of bursae (pers. obs.). Calappids are presumably the sister group of cancrids and atelycyclids, and are probably closer to their common ancestor (Rice, 1980). Dissection of a female *Calappa ocellata* revealed genitalia similar to that of *Cancer* species but no trace of a bursa (pers. obs.). Thus, the bursa seems to be an apomorphism of the *C. magister* lineage [where the fossil record goes back to the Miocene (Nations, 1979)].

(4) *Protracted female receptivity.* Hypotheses to explain post-copulatory mate guarding by male crabs invoke protection of the male's reproductive investment. In addition to preventing the introduction of rival ejaculates to the spermathecae (which in the case of *C. magister* is accomplished by the sperm plug), guarding confers protection to soft females from predators (Hartnoll, 1969; Hazlett, 1975; Nelson and Hedgecock, 1977; Wilber, 1989) and from female-female interactions, which in this species are particularly aggressive and often lethal (Jacoby, 1983; pers. obs.). Post-copulatory protection arguably contributes to the fitness of the female; therefore, selection is expected to favor traits that elicit or prolong it. Observations on *C. gracilis,* a closely related species in which there is no bursa, indicate that males will attempt copulation with still soft females that have mated previously with another male; the "secondary" male does not show normal post-copulatory behavior and deserts the female soon after copulation (Orensanz *et al.,* 1995). Our data on the behavior of "primary" and "secondary" male mates in *C. magister* are limited, but it might be hypothesized that effective reception of sperm in the bursa from "secondary" males, together with a plug that does not interfere with intromission, could elicit a guarding response from "secondary" males, even when their sperm (being diverted to the bursa) are not used later in fertilization.

Although we conclude that the most obvious hypothesis for the function of the bursa, sperm storage, is unlikely, our conclusion is based on indirect evidence. Before this hypothesis can be confidently rejected, the role the bursal sperm play in fertilization, if any, must be determined. We are currently investigating this aspect of the mating system in *C. magister.*

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A Chemical Cue Induces Settlement of Sydney Rock Oysters, *Saccostrea commercialis,* in the Laboratory and in the Field

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Abstract. The physical and chemical nature of a substratum's surface, including the presence of dissolved compounds or a bacterial film, may induce the settlement of invertebrate larvae. Based on previous observations of (1) enhanced recruitment of Sydney rock oysters (*Saccostrea commercialis*) on concrete surfaces and (2) high alkalinity at the surface of concrete due to the leaching of calcium hydroxide ($Ca(OH)_2$) from the cement, experiments were designed to test the hypothesis that a surface containing $Ca(OH)_2$ enhances settlement of oysters. The effect of a biofilm on settlement of Sydney rock oysters was also tested. The results obtained from the field experiment differed slightly from those obtained in the laboratory. In both the laboratory and the field, $Ca(OH)_2$ had a positive effect on settlement. No significant effect of a biofilm on settlement on concrete substrata was observed in either experiment; a positive effect of a biofilm on substrata made with sand only or on sandstone was seen in both the laboratory and the field. For the substrata made with $Ca(OH)_2$ and sand, however, the effect of a biofilm was positive in the field but negative in the lab. Although the results from the lab and field experiments were similar, the discrepancy was crucial in accepting or rejecting one of the hypotheses under test. The results of the study demonstrated that (1) the effects of a biofilm on settlement vary with the substratum and (2) these oysters can respond to a chemical cue ($Ca(OH)_2$) in the field. The ecological implications of the ability of larvae to select a habitat in response to chemical cues are discussed.

Introduction

With the emergence (or re-emergence, see review in Young, 1990) of supply-side ecology, considerable emphasis is placed on the importance of the supply and settlement of marine invertebrate larvae to the patterns of distribution and abundance of juveniles and adults (Underwood and Denley, 1984; Underwood and Fairweather, 1989). Several experimental studies have now clearly demonstrated how ecologically important settlement can be in shaping such patterns (*e.g.*, Connell, 1985; Gaines and Roughgarden, 1985; Bushek, 1988; Raimondi, 1988a, 1991; Minchinton and Scheibling, 1991). Therefore, there are important ecological implications for the potential ability of marine invertebrate larvae to exercise behavioral selection of habitat at settlement, when the chemical and physical attributes of a substratum may influence such behavior.

Settlement of larvae in response to specific chemical cues occurs in many species (see reviews by Crisp, 1974; Pawlik and Hadfield, 1990; Young, 1990; Pawlik, 1992; note also the important early work by Crisp and Meadows, 1962, 1963, and Knight-Jones, 1951, 1953). Chemicals that induce settlement behavior or metamorphosis are very diverse and include γ-aminobutyric acid (GABA) (*e.g.*, Morse, 1985; Pearce and Scheibling, 1990), L-3,4-dihydroxyphenylalanine (L-DOPA) (Coon *et al.*, 1985), cholines (Hadfield and Scheuer, 1985; Hadfield and Pennington, 1990), ammonia (Coon *et al.*, 1990), sulphide (Cuomo, 1985), and excess potassium ions (Nell and Holliday, 1986; Yool *et al.*, 1986).

Experiments with larvae that exhibit gregarious settlement behavior (settlement in response to the presence of conspecifics) have also resulted in the identification of chemical signals triggering settlement (see review in Burke, 1986), including free fatty acids (Pawlik *et al.*, 1991), 2,6-di-*tert*-butyl-4-methylphenol (DBMP) and 3-isobutyl-1-methylxanthine (IBMX) (Jensen and Morse, 1990) and glycyl-glycyl-L-arginine and other peptides (Zimmer-Faust

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and Tamburri, 1994). Another group of chemical cues that enhance settlement of marine invertebrates is in the form of biofilms or bacteria colonizing submerged surfaces (Brancato and Woollacott, 1982; Kirchman and Mitchell, 1983; Weiner *et al.,* 1989; Johnson *et al.,* 1991; Tamburri *et al.,* 1992; Todd and Keough, 1994; Keough and Raimondi, 1995).

Notwithstanding such extensive evidence of the ability of larval invertebrates to differentiate chemical cues, the relative importance of this ability in dictating patterns of settlement in natural field situations is unclear. Much of the evidence for the ability of larvae to respond to chemical cues comes from laboratory experiments, with no demonstration of whether results can be extrapolated to infer that larvae actually exercise habitat selection using such cues.

Reasons for the relative lack of field studies include the difficulty in predicting the time and place of settlement or the difficulty in finding newly settled individuals in the field. Recognizing these problems and the limitations of laboratory experiments performed in still seawater, several researchers have advocated the use of flume experiments in the laboratory in an attempt to simulate the hydrodynamics experienced by larvae in the field (Butman, 1986; Hadfield, 1986; Butman, 1987; Pawlik *et al.,* 1991; Turner *et al.,* 1994).

Although experiments done in controlled laboratory flow regimes allow determination of specific hydrodynamic conditions that would permit active habitat selection, they still cannot be considered as a substitute for field conditions, because many other factors such as light, other organisms, gradients of temperature and salinity, and food in the plankton may play a role in modifying behavior of larvae at settlement.

Only a few experiments strongly support the hypothesis that larvae can respond to specific chemical cues in the field (see Raimondi, 1988b; Jensen and Morse, 1990; Morse *et al.,* 1994). Jensen and Morse (1990) and Morse *et al.* (1994) provided the only demonstrations in both the laboratory and the field of the effectiveness of an identified chemical cue in triggering settlement and metamorphosis [although the field experiment in Morse *et al.* (1994) was done with laboratory-reared larvae].

The present study was designed to determine the effectiveness of a chemical cue in triggering settlement of the Sydney rock oyster, *Saccostrea commercialis,* in a laboratory and a field experiment. The field experiment was done to assess the relevance of the results obtained in the laboratory. Historical information concerning the seasonal time and place of settlement of these oysters (S. McOrrie, unpub. data) made it possible to estimate settlement in the field. The experiments in the lab and the field were designed to be as comparable as possible to determine the likelihood of being able to extrapolate laboratory results to the field situation.

Although laboratory experiments are appropriate where a commercial oyster industry depends on hatchery-reared oysters, the commercial oyster farming industry in New South Wales, Australia, depends on natural settlement of oysters on intertidal structures. Virtually no experiments have been done in an effort to understand their larval settlement behavior (but see Holliday *et al.,* 1993).

The following series of observations led to the development of the hypotheses concerning a chemical cue tested in this study. First, Anderson and Underwood (1994) found that *S. commercialis* recruited in greater numbers on concrete surfaces than on wood, fiberglass, and aluminium substrata. Second, the seawater at the surface of concrete used in Anderson and Underwood (1994) had a mean (±S.E.) pH of 9.24 (±0.07), because of leaching of calcium hydroxide ($Ca(OH)_2$) from the cement in concrete, while seawater at the surfaces of the other substrata did not show a change in pH relative to surrounding seawater. Third, in laboratory experiments with larvae of a related oyster, the Pacific oyster *Crassostrea gigas,* settlement behavior was induced by ammonia (NH_3) and other weak bases. I therefore hypothesized that there should be enhanced settlement of Sydney rock oysters on concrete surfaces, and further that it is the $Ca(OH)_2$ content in cement that is the chemical cue causing this response.

In addition, there is substantial evidence from laboratory work on the Pacific oyster, *C. gigas,* and the American oyster, *C. virginica,* that biofilms trigger settlement in these species (Weiner *et al.,* 1989; Bonar *et al.,* 1990; Coon *et al.,* 1990; Tamburri *et al.,* 1992). In particular, Coon *et al.* (1990) reported the induction of settlement behavior by NH_3 produced by bacteria. Also, Bonar *et al.* (1990) noted that the equilibrium between NH_3 and NH_4^+ in seawater is sensitive to pH such that a downward shift (*e.g.,* from pH = 8.0 to 7.6) favors the presence of NH_4^+ and greatly decreases the effectiveness of NH_3 in triggering settlement behavior.

From this line of evidence, I hypothesized that biofilms may enhance settlement of *S. commercialis* and, additionally, that there may be an interaction between the pH at a surface and the presence of a biofilm such that the effect of these two factors combined would be greater than the effect of either factor alone. Expressed another way, a relatively high pH at a surface would effectively enhance the ability of a biofilm to increase larval settlement.

Materials and Methods

Treatments

To isolate the effect of $Ca(OH)_2$, it was necessary to develop a substratum comparable to concrete in terms of its content of $Ca(OH)_2$ (and resulting pH at its surface), but which did not contain all of the other compounds in cement. Substrata were made by spreading non-toxic hydrophilic epoxy cement (Megapoxy HT) on one side of quarry tiles (11 × 11 cm) and coating this with dry mixtures of varying proportions of $Ca(OH)_2$ and sand (the same type of sand used in making concrete substrata). The pH of seawater at the surface of concrete and of substrata made on tiles was measured after drawing up 2 ml of fluid into a micropipette being dragged slowly across each surface.

It was found that seawater at the surface of substrata made with a mixture in the ratio of 3 parts sand to 1 part $Ca(OH)_2$ by weight (hereafter called E3) closely matched the pH of seawater at the surface of concrete (average pH = 9.24) for the relevant period of these experiments. Substrata were also made with epoxy and sand only (ES). The pH at the surface of these substrata did not differ from that of surrounding seawater (average pH = 8.19).

Tests for the effect of $Ca(OH)_2$ on larval settlement could be made by comparing settlement on E3 with that on ES. Tests for the effects of concrete on larval settlement that were not caused by $Ca(OH)_2$ could be made by comparing settlement on concrete with that on E3. In addition, to test for the effects of a biofilm and its potential interaction with the pH of surfaces, each of E3, ES and concrete surfaces were exposed to seawater to develop a biofilm. These surfaces were "aged" in the containers in the one-way flow-through system described below (see *Laboratory experiment*) for 20 days prior to the beginning of the laboratory experiment.

The field experiment included an additional treatment of sandstone substrata along with concrete, E3 and ES as used in the lab experiment. This was to control for any effect of the epoxy resin and to include in the field experiment a natural surface that would be encountered by oyster larvae in estuaries in New South Wales. Four more treatments in the field experiment consisted of each of concrete, sandstone, E3 and ES having developed a biofilm for twenty days in the same manner as for the lab experiment. Biofilms for the field experiment were developed in the lab to make this factor as comparable as possible between the two experiments. Treatments that were not exposed to seawater prior to the beginning of experiments will be referred to as "substrata without a biofilm," even though some colonization by microorganisms may have occurred by exposure to seawater during experiments.

Concrete and sandstone substrata measured 10 × 10 cm; substrata made with quarry tiles measured 11 × 11 cm (the commercial standard for quarry tiles). Thus, an area of 10 × 10 cm was sampled from all substrata when counting settled larvae in either the field or the laboratory experiment.

Laboratory experiment

The laboratory experiment was designed so that compounds leaching from various surfaces could not affect other treatments. A one-way flow-through system was used where a single source of seawater (filtered coastal seawater with a salinity of 35‰) was pumped into a central manifold pipe then diverted separately into containers that were each an independent replicate ($n = 4$ containers per treatment). Larvae were fed during the experiment by adding 10,000 algal cells per ml (*Chaetoceros calcitrans, Isochrysis galbana* and *Pavlova lutheri*) to the source of seawater.

Each container consisted of a section of 235 mm-diameter PVC pipe (length = 14 cm) fitted with a 150-μm mesh screen and lowered into a 10-l plastic bucket without being totally submerged (Fig. 1). Seawater flowed through the mesh at the bottom of each container at a rate of *ca.* 1.5 l/h into the buckets, then out over the edge of the buckets into a water bath surrounding the buckets (maintained at 23°C), and finally to waste (Fig. 1). There was no recirculation of seawater, so no confounding of water from one replicate to another.

Aeration was added to each container to maintain uniform circulation of seawater and to prevent larvae from clumping against the mesh. One panel was suspended in each container with its settling surface facing downwards because it has been found that undersurfaces attract the greatest settlement of oysters (Ritchie and Menzel, 1969; Michener and Kenny, 1991).

Approximately 5,000 competent larvae (>212 μm) of *S. commercialis* reared in the laboratory were placed in each container. The number of larvae settling on each panel was counted after 24 hours and then again each day for five more days.

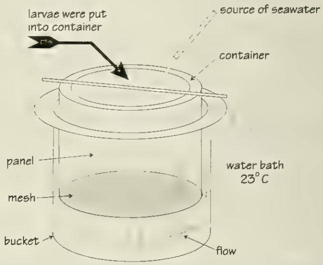

Figure 1. Diagram of one-way flow-through system used in the experiment. Seawater flowed into a PVC container that contained the larvae, down through 150-μm mesh into a 10-l bucket, then flowed over the lip of the bucket into a water bath and then to waste. One replicate substratum was suspended in each container. There was no recirculation of seawater and thus no confounding of replicates.

Table I

Analysis of variance of number of oysters settling after six days in the laboratory experiment on concrete, E3 or ES (see description of substrata in text) either with or without a biofilm (n = 4)

Source	Df	Mean square	F	P
Substratum	2	8710807	39.86	<0.001***
Biofilm	1	433628	1.98	>0.17
Substratum × Biofilm	2	949440	4.34	<0.03*
Residual	18	218514		
Total	23			

Substratum and Biofilm are fixed factors; data are untransformed; variances were homogeneous by Cochran's test ($P > 0.05$).

Field experiment

The field experiment was done at two sites (separated by > 300 m) in Salamander Bay at the south side of the Port Stephens estuary, New South Wales. Previous data taken biweekly of larval supply and settlement throughout the outer basin of this estuary for the period 1985–1991 (S. McOrrie, unpub. data) and data on recruitment in Salamander Bay for 1993 and 1994 (Anderson, unpub. data) were used to predict where and when oysters would settle in the 1995 season.

The experiment used existing structures at commercial oyster leases in Salamander Bay to support the substrata. These structures, situated throughout the Bay including at the two sites used for the experiment, consisted of two parallel beams (5 × 2.5 cm in thickness) of tar-covered hardwood, each several meters long and situated approximately 1 m apart. The beams were supported by several timber posts embedded in the sand such that the resulting tidal height of the beams was about 0.5 m above Spring Low Water.

There were 32 panels (4 replicates of each of 4 substrata, ± biofilm) at each site. These were attached in random order, 26.6 cm apart, to eight 180-cm long, tar-covered hardwood sticks (2.5 × 2.5 cm) using self-tapping stainless steel screws (four per stick). Sticks were placed perpendicularly across the two parallel beams with surfaces of panels facing downwards between the beams. The sticks were attached to the beams with plastic cable ties approximately 20–30 cm apart.

The distance between sticks was similar to the distance between panels on a single stick. This design resulted in a randomized array of 4 × 8 panels at each site, so that particular treatments could not be correlated with hydrodynamic conditions that may have varied across the array.

Substrata were placed in the field on 15 April 1995 and retrieved on 24 April 1995, when the number of oysters settled on each substratum was recorded.

Results

Laboratory experiment

For substrata without a biofilm, concrete had significantly greater numbers of oysters than E3, which in turn had significantly greater numbers of oysters than ES (SNK tests, $P < 0.05$, Table I, Fig. 2A). Thus, $Ca(OH)_2$ had a positive effect on settlement, but this effect was not large enough to explain the even greater response of oyster larvae to concrete substrata.

The effect of the presence of a biofilm was significant and varied with the substratum (significant Substratum × Biofilm interaction term, Table I, Fig. 2A). On ES, the presence of a biofilm significantly increased settlement (SNK test, $P < 0.05$), while settlement decreased, but not significantly, when a biofilm was present on concrete or E3 (SNK tests, $P < 0.05$, Table I, Fig. 2A). Also, the effect of different substrata on settlement changed with the presence of a biofilm (Fig. 2A). Concrete still had significantly greater numbers of oysters than ES or E3, but there was no significant difference in numbers of oysters on ES and E3 (SNK tests, $P < 0.01$, Table I, Fig. 2A).

Field experiment

In the analysis of variance (ANOVA) of the field data, there were heterogeneous variances even after transformation of the data to $x' = \log_e(x + 1)$ (Cochran's test, $P < 0.01$). Although ANOVA is fairly robust to violations of the assumption of homogeneity of variances in a test of this size, some caution should be used in interpreting significant results (Cochran, 1947; Underwood, 1981).

Notwithstanding the significant Site × Substratum interaction, the effects of different substrata were reasonably

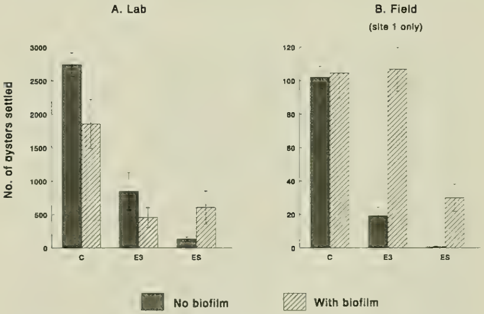

Figure 2. Mean (±S.E.) number of oyster larvae settled on substrata in (A) the laboratory experiment and (B) the field experiment (site 1 only) either with or without a biofilm ($n = 4$): C = concrete, E3 = substratum made with epoxy and 3 parts sand to 1 part $Ca(OH)_2$, ES = substratum made with epoxy and sand only. Note the difference in scale of the y-axes.

consistent across the two sites (Table II, Fig. 3). There was greater overall settlement at site 2 (note the difference in scale of the y-axes, Fig. 3) and the Site × Substratum interaction was caused by this being significant for E3 and ES, but not for concrete or sandstone (SNK tests, $P < 0.01$ and $P > 0.05$, respectively).

The effect of a biofilm on substrata was consistent across sites (non-significant Site × Biofilm interaction term, Table II). The presence of a biofilm did have a significant effect that varied with the substratum, as in the laboratory experiment (significant Substratum × Biofilm interaction term, Table II). The presence of a biofilm significantly increased settlement of oysters on all substrata except concrete, for which it had no effect (SNK tests, $P < 0.05$, Fig. 3).

For substrata without a biofilm, the results were similar to the results obtained in the laboratory (Fig. 2A, B). There was significantly greater settlement on concrete than E3, which had greater settlement than ES (SNK tests, $P < 0.05$). Settlement on sandstone was significantly less than on concrete or E3, but greater than on ES (SNK tests, $P < 0.05$, Fig. 3).

On substrata with a biofilm, the results in the field differed somewhat from those in the laboratory (Fig. 2A, B). There was no significant difference between E3 and concrete, and both had significantly greater settlement than ES (SNK tests, $P < 0.05$, Fig. 3). Once again, sandstone had fewer settlers than on concrete or E3, but more than on ES (SNK tests, $P < 0.05$, Fig. 3).

Discussion

Laboratory vs. field experiment

In both experiments, on substrata without a biofilm, concrete had greater settlement of oysters than E3, and E3 had greater settlement than ES. This indicated that, as a chemical cue, $Ca(OH)_2$ had a positive effect on settlement and that larvae can respond to this cue in laboratory and natural environments.

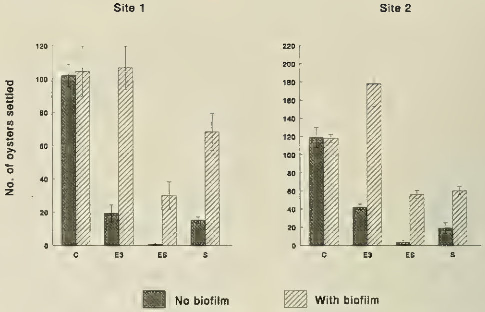

Figure 3. Mean (±S.E.) number of oysters settled on substrata at site 1 and site 2 in the field experiment either with or without a biofilm ($n = 4$): C = concrete, E3 = substratum made with epoxy and 3 parts sand to 1 part $Ca(OH)_2$, ES = substratum made with epoxy and sand only, S = natural sandstone. Note the difference in scale of the y-axes.

Table II

Analysis of variance of number of oysters settling after nine days in the field experiment on concrete, E3, ES or sandstone (see description of substrata in text) either with or without a biofilm at each of two sites (n = 4)

Source	Df	Mean square	F	P
Site	1	2.46	13.67	<0.001***
Substratum	3	18.46	30.86	<0.01**
Biofilm	1	34.92	361.49	<0.04*
Site × Substratum	3	0.60	3.35	<0.03*
Site × Biofilm	1	0.10	0.54	>0.46
Substratum × Biofilm	3	5.99	294.91	<0.001***
Site × Sub × Biofilm	3	0.02	0.11	>0.95
Residual	48	0.18		
Total	63			

Substratum and Biofilm are fixed factors and Site is a random factor. Data were transformed to $x' = \log_e(x + 1)$, but variances were still heterogeneous by Cochran's test ($P < 0.01$).

Also, on surfaces without a biofilm, the field experiment showed greater settlement of oysters on sandstone substrata than on ES, indicating that the the epoxy glue on E3 and ES substrata probably had some negative effect on settlement. This negative effect, however, was still not great enough to explain the discrepancy between concrete and E3 without a biofilm. It appears, therefore, that although $Ca(OH)_2$ enhances settlement, there are other components of concrete, chemical or physical, that may also increase settlement of oysters.

In addition, both experiments demonstrated no significant effect of a biofilm on concrete and a positive effect of a biofilm on ES. The significant Substratum × Biofilm interaction emphasizes that biofilms are complex, dynamic assemblages. The variation in the effect of a biofilm on different surfaces could be due to the fact that the composition of bacteria and micro-organisms differed on different substrata (Edyvean *et al.*, 1985; Maki *et al.*, 1990) or the interaction of a biofilm with a surface produces a unique chemical or physical signature that differs on different surfaces (Mihm *et al.*, 1981). Maki *et al.* (1990) and Kirchman and Mitchell (1983) suggested that larvae respond to extracellular polymers on the surface of bacteria, and that these exopolymers have different stereochemical orientations when bacteria are attached to different surfaces.

The single discrepancy between the results from the laboratory and those from the field was the difference in the effect of a biofilm on E3 substrata. In the laboratory, the presence of a biofilm on E3 significantly decreased settlement, while its presence in the field significantly increased settlement of oysters, even to the point of equalling (site 1) or exceeding (site 2) settlement on concrete. Although this was really the only major difference between the results of the two experiments, it is one that has great bearing on an important hypothesis in this study: namely, that the combined effect of $Ca(OH)_2$ and the presence of a biofilm would be greater than either effect alone. Given only the results of the laboratory experiment, this hypothesis would be rejected, whereas the field results substantially supported it.

This discrepancy between the two experiments suggests that even when biofilms are developed for the same period under the same conditions, the short-term variation in such biofilms can cause important differences in larval settlement. Further research is needed to characterize these biofilms and to test hypotheses about the effects of specific components of the films on larval settlement, and in particular, to determine what component may have caused such a dramatic difference in settlement in the field *vs.* in the laboratory on E3 surfaces.

There are several reasons why one might expect the field results to differ from results obtained in the laboratory. Larvae in the laboratory were supplied in great numbers (approx. 5,000 per container) and were captive in containers. Differences in settlement could be due to differences in sheer numbers of oysters settling (note difference in the scale of the y-axes, Fig. 2), or some aspect of gregarious settlement (Hidu *et al.,* 1978; Tamburri *et al.,* 1992). One would expect an increase in the effect of gregariousness (if it exists for *S. commercialis*) between larvae and recently settled individuals in closed containers in the laboratory, which would amplify the size of any initial differences in settlement among substrata (Gotelli, 1990).

There were also differences in the duration and timing of experiments. The experiment in the laboratory was done over six days in January 1995; the field experiment was done over nine days in April 1995. Although biofilms for both experiments were developed in the lab, there could have been seasonal differences in the composition of micro-organisms colonizing surfaces (Anderson, 1995). In addition, biofilms can form rapidly and micro-organisms could have modified existing biofilms on surfaces, as well as colonized clean surfaces, during the period of exposure to larvae. Substrata in the field experiment may have developed a more diverse and complex biofilm during their exposure that differed from that in the laboratory. Keough and Raimondi (1995) found no difference in the response of larvae of many taxa to films in the field versus the laboratory. They and others (*e.g.,* Maki *et al.,* 1988, 1990) have, however, found some effect on settlement of the age of films.

There would also have been differences in the hydrodynamics of the two experiments. One would expect that greater flow velocities, due to tides and currents, would occur in the field than in the lab, and that in these higher velocities, larvae may not be able to exercise habitat selectivity (Hannan, 1984). Indeed, Butman (1986) argues that most near-bottom water flows exceed maximum larval swim speeds and further that the effectiveness of chemical cues would diminish with an increase in flow velocity, becoming very dilute within a short range (Butman, 1987).

Despite the numerous potential differences between experiments in the laboratory and the field, *S. commercialis* larvae responded consistently to 5 of the 6 treatments (3 substrata, ± biofilm) presented similarly in the two environments. Results from this study, Jensen and Morse (1990) and Morse *et al.* (1994) suggest that natural chemical cues found to trigger settlement in laboratory experiments may well be acting to determine the selection of habitat of larvae in the field. As a note of caution, however, the hypothesis of enhanced settlement in the presence of a biofilm and increased $Ca(OH)_2$ was rejected by the laboratory experiment but was supported by results in the field. This emphasizes that there is no substitute for field experiments where information about larval settlement in the field is required.

Mechanism of the chemical cue

Why should *S. commercialis* respond to the presence of $Ca(OH)_2$ and a relatively high pH on a surface in the field? This compound is not normally present in this form in the marine environment. Natural changes in the pH of seawater within the approximate range of 7.5–8.5 are largely determined by the bicarbonate/borate concentration and are due to photosynthesis and respiration of marine organisms (Parsons *et al.,* 1984). Low pH values are reflected in high concentrations of carbon dioxide. Conversely, areas of high photosynthesis also have high concentrations of oxygen and higher pH values (Parsons *et al.,* 1984). Oyster larvae may have a natural response to settle in areas of relatively high pH as a cue to areas within an estuary with high phytoplankton productivity as a food source and abundant oxygen for growth.

This is consistent with the results of Coon *et al.* (1990) who found that NH_3 and other weak bases trigger settlement behavior in the Pacific oyster, *Crassostrea gigas.* They suggested that these substances act by raising intracellular pH by penetrating the cell membrane as an uncharged species, then re-protonating in the cytoplasm (Bonar *et al.,* 1990; Coon *et al.,* 1990). The presence of excess $(OH)^-$, as in this study, could act directly to increase intracellular alkalization.

It may be, however that an excess of Ca^{2+} is responsible for changing the ionic potential across the larval cell membrane, triggering a cascade of neural activity in the larvae, resulting in metamorphosis. Yool *et al.* (1986) suggested the alteration of membrane ion conductance to be the mechanism whereby excess K^+ ions induced settlement in four marine invertebrate species. They further observed that *Phragmatopoma californica* larvae metamorphosed in excess Ca^{2+} medium, suggesting that Ca^{2+} may play the same role as K^+ in signal transduction. Nell and Holliday (1986) found that excess K^+ triggered settlement in *S. commercialis,* so it is not unlikely that Ca^{2+} in this experiment acted via a similar mechanism to trigger the settlement response.

Jensen and Morse (1990) suggested further that other chemical inducers, including free fatty acids, DBMP and IBMX, could act via such a mechanism of ionic depolarization in triggering settlement of *Phragmatopoma californica* by elevating levels of intracellular cyclic AMP. Morse (1985) proposed a single mechanism for the induction of settlement of invertebrate larvae by suggesting that neurotransmitter-mimetic inducers, such as GABA, choline, and DOPA, act by binding to receptors on larval chemosensory membranes, resulting in depolarization and triggering of the metamorphic pathway. He added that induced changes in cyclic AMP and calcium or potassium concentrations could directly affect membrane potentials and settlement.

In contrast, Pawlik (1990) argued against there being sufficient evidence for the existence of a single metamorphic pathway for different species. No specific mechanisms were investigated by the experiments reported here, but results were not inconsistent with the above models suggested by Morse (1985), Jensen and Morse (1990), Yool *et al.* (1986) and Bonar *et al.* (1990).

Habitat selectivity

Butman (1987) discussed the evidence in favor of marine invertebrate larvae being able to actively select habitats in contrast to the idea that larvae are passively deposited on substrata (*i.e.,* the "passive deposition hypothesis"). In several instances of larval "selection" of particular locations due to physical characteristics, the hydrodynamical processes associated with such physical characteristics were consistent with a similar "selection" by inanimate particles, so the passive deposition hypothesis could not be rejected (Eckman, 1983; Hannan, 1984). Nevertheless, correlative field observations (Strathmann and Branscomb, 1979; Hudon *et al.,* 1983; Chabot and Bourget, 1988; LeTourneaux and Bourget, 1988) and manipulative field experiments (Wethey, 1986; Raimondi, 1988a; Michener and Kenny, 1991; Walters and Wethey, 1991; James and Underwood, 1994) provide evidence that larvae can select a habitat by virtue of physical characteristics.

Although chemical cues affect larval settlement in many laboratory experiments (reviewed in Pawlik, 1992), I have found only three published experimental studies demonstrating a real effect of a chemical cue on larval settlement in the field (Raimondi, 1988b; Jensen and Morse, 1990; Morse *et al.,* 1994). The passive deposition hypothesis was also clearly rejected by the present study. Oyster larvae responded positively in the field to the presence of $Ca(OH)_2$, to concrete, and to the presence of a biofilm. Furthermore, these responses were similar to responses of larvae in the laboratory.

In his review, Pawlik (1992) stated that it is still "unclear whether any of these [chemical cues] induce settlement under natural conditions," and advised that "greater effort should be devoted to testing stimulatory substrata or compounds under more realistic flow regimes, *i.e.,* in flumes or in the field." This study followed his advice and demonstrated that oysters of *S. commercialis* can actively select a substratum in the field according to its chemical composition. The ecological consequences of marine invertebrate larvae being able to exercise habitat selectivity in response to chemicals in the field are very important because their settlement can determine their patterns of distribution and abundance as adults.

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Conspecific Recognition and Host Choice in a Pea Crab, *Pinnixa chaetopterana* (Brachyura: Pinnotheridae)

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Abstract. The pea crab *Pinnixa chaetopterana* (Brachyura: Pinnotheridae) lives in the tubes of the host polychaetes *Chaetopterus variopedatus* and *Amphitrite ornata.* While attraction to host odor cues is common in symbiotes, *P. chaetopterana* resident in *Chaetopterus* tubes apparently show no attraction to water-borne cues from either host. Because crabs resident in *Amphitrite* tubes are significantly reduced in their maximum body size and thus clutch size, switching between hosts should occur. We therefore tested the hypothesis that the crabs use odor cues from conspecifics or from the competing crab *Polyonyx gibbesi* (Anomura: Porcellanidae) when locating a new host. Crabs collected from *Amphitrite* are significantly attracted to *Chaetopterus* hosts which contain a mixed sex pair of conspecifics. Females show a strong tendency to avoid hosts containing *Polyonyx,* but males do not. Neither sex is attracted to unoccupied *Chaetopterus* or *Amphitrite* hosts, but both are significantly attracted to isolated conspecifics. This attraction could serve as a reliable means of locating suitable new hosts when switching hosts in the field. Attraction to conspecific odors would also increase the chances of finding a suitable mate already present in a new host.

Introduction

Symbioses are common in terrestrial, fresh water, and marine environments. The importance and prevalence of symbioses in a given system can be influenced, however, by several factors: fitness payoffs to both hosts and their symbionts (Roughgarden, 1975; Keeler, 1985), spatial patterning or density of the host population (Blower and Roughgarden, 1989), and the ability of symbionts to recognize suitable hosts and initiate the symbiotic relationship (Dimock and Davenport, 1971; Derby and Atema, 1980; van Alphen and Janssen, 1982).

Host recognition abilities can be important in different life stages of a symbiotic species. Often, hosts are chosen by mobile, dispersive larval or juvenile stages that initiate permanent relationships and maintain them for the remaining life of the settler. In organisms that freely switch hosts, adult stages also require the ability to recognize hosts. Host switching may occur following the death of a host, during the search for a mate, or when the maximum attainable size and reproductive output of an adult symbiont is limited by the resources available in the association with a particular host individual (Castro, 1978; Wirtz and Diesel, 1983).

Common marine examples of symbiosis are found in the brachyuran decapod family Pinnotheridae. There are over 100 species of these crabs living in both facultative and obligate relationships with a wide variety of hosts including bivalves, polychaetes, holothurians, ascidians, echinoids, and gastropods. Some pinnotherid species choose hosts during early life stages and apparently metamorphose into the adult form while resident within the host (Christensen and McDermott, 1958; Wells and Wells, 1961; Eidemiller, 1969). A number of species, however, change hosts as adults (Pearce, 1966; Bell, 1984) and are attracted to odors emanating from potential new hosts (Sastry and Menzel, 1963; Gray *et al.,* 1968; Stevens, 1990).

One exception to this attraction-to-host-odor trend is the pinnotherid species *Pinnixa chaetopterana,* which is commonly found (generally in heterosexual pairs) inside the tubes constructed by the polychaetes *Chaetopterus*

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variopedatus and *Amphitrite ornata,* as well as less commonly in the tubes of the polychaetes *Clymenella torquata* and *Loimia viridis* and the bivalve *Tagelus plebeius* (Ruppert and Fox, 1988). Adult crabs collected from *Chaetopterus* show no net displacement towards an odor source from their host or from *Amphitrite,* although they do display increased rates of random turning when inside a host odor plume (Davenport *et al.,* 1960).

It is surprising that adult *Pinnixa chaetopterana* are not clearly attracted to water-borne odor cues from their hosts because some marked individuals switch hosts both in the laboratory and in the field (Grove, pers. obs.). Host switching, especially from *Amphitrite* to *Chaetopterus,* may be an adaptive behavior because *Amphitrite* tubes are smaller than *Chaetopterus* tubes. The body sizes of several symbiotic crustacean species can be correlated with host size (Knowlton, 1980; Adams *et al.,* 1985; Duffy, 1992). The assumption that male and female *P. chaetopterana* have significantly smaller body sizes and smaller clutch sizes when resident in *Amphitrite* as compared to crabs resident in *Chaetopterus* was therefore explicitly tested.

In addition, we tested the hypothesis that adult *Pinnixa chaetopterana* respond to odor cues eminating from other host occupants when searching for a host. Responses to conspecific odors can be positive, increasing opportunities for mating (Gleeson, 1980; Eller *et al.,* 1984) or the potential for defense against predators (Zimmer-Faust *et al.,* 1985; Gerhart, 1986), or negative, as occurs when conspecifics compete for host resources (McNeil and Quiring, 1983; Bell, 1984).

P. chaetopterana competes for possession of *Chaetopterus* hosts with the anomuran crab *Polyonyx gibbesi* (Family Porcellanidae), and adults of the two species are rarely found together in the same host tube. In the field, a crab searching for a new host should generally encounter both host odor cues and odor cues from one of the two crab species simultaneously because less than 6% of *Chaetopterus* hosts are not occupied by either species of crab (Enders, 1905). Thus we tested the reaction of *P. chaetopterana* to odor cues from hosts containing either conspecifics or *Polyonyx gibbesi* as well as the reaction to crabs without hosts. The possibility that there are tactile cues associated with host tubes was not investigated, but these are unlikely to be the only cue types because the average distance between *Chaetopterus* hosts in sites in the study habitat is approximately 4.5 m (Grove, unpub. data).

Materials and Methods

Pinnixa chaetopterana and *Polyonyx gibbesi* and their host polychaetes *Amphitrite ornata* and *Chaetopterus variopedatus* were collected at regular intervals over a three-year period (1992–1994) from intertidal mudflats in Debidue Creek, North Inlet, South Carolina (33° 20′ N, 79° 10′ W). Worms and crabs were removed from the tubes and wet weights to the nearest 0.01 gram were obtained. The body length and width of the crabs were measured to the nearest 0.1 millimeter using calipers. At least 60 crabs of each sex from each host were measured during this period.

Any gravid females of *Pinnixa* collected were frozen on dry ice, preserved in 10% formalin, and stored in 70% ethanol. The number of eggs per clutch was subsequently enumerated and the crabs were dried to constant weight at 60°C to determine the relationship between female body size and clutch size.

The maximum internal tube diameters of six *Chaetopterus* tubes inhabited by *Pinnixa chaetopterana* were obtained by sequentially removing 2-cm-long segments of the tubes with a razor blade and measuring the diameter of each segment with calipers. The volumes of empty *Chaetopterus* tubes previously inhabited by *Pinnixa* or *Polyonyx gibbesi* (5 tubes per crab species) were determined by filling the tubes with water and then draining the tubes into a graduated cylinder.

All laboratory experiments were run in the fall of 1993 and the spring of 1994 in a six-chambered choice apparatus modified from Bartel and Davenport (1956) (Fig. 1). In all experiments, equal water flow (300 ml/h/chamber) was forced into the six radial chambers by a peristaltic pump (Watson-Marlow, Wilmington, Massachusetts) and drained through a standpipe in the central chamber. Flow visualization studies using fluorescein indicated that there was mixing of water from the radial chambers around the standpipe, but there were distinctive and separate plumes near the entrance to each radial chamber.

The central chamber had a diameter of 30 cm and was lined with washed foundry sand (220 μm mean grain size) to a depth of 2 cm. PVC pipes (17 mm dia.) leading to the outer chambers opened at the sand surface. Water volume in the central chamber was 2.8 liters, while that in each of the six surrounding chambers was 650 ml. All experiments were performed in a darkened environmental chamber at 23 ± 2°C. Natural seawater (34 ± 1 ppt) was filtered through a 3-μm bag prior to use.

Pinnixa chaetopterana used in the experiments were collected from tubes of *Amphitrite ornata* in Debidue Creek. *Pinnixa* specimens escaped vertically down the host tube, preventing the collection of large sample sizes. Crabs were transported to the laboratory, weighed and measured, and allowed to acclimate in individual containers for at least 48 h prior to use. No gravid female crabs were used in any of the experiments.

The tubes of *Chaetopterus variopedatus* were gently slit open using a razor blade, and the worm and any crab occupants removed and weighed. The worm and crabs

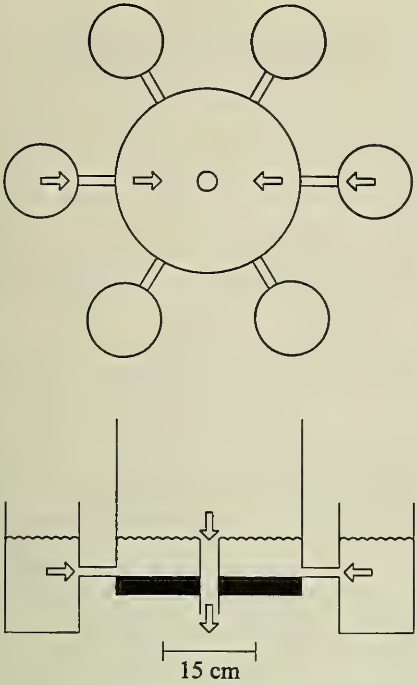

Figure 1. Six-chambered choice apparatus used in all experiments. Arrows indicate direction of water flow.

appropriate for the experiment being run were replaced, and the tube slit was sewed shut using cotton thread. Worms were allowed to acclimate in running seawater for 72 h after handling before further use. The mean weight of *Chaetopterus* used was 13.05 ± 3.56 g.

Host by conspecific experiment

In this experiment, conditioned seawater was pumped into the outside chambers from three treatment reservoirs, with water from a given reservoir pumped into two chambers located 180° apart. The reservoirs contained: (1) filtered seawater alone (control treatment), (2) filtered seawater plus two *Chaetopterus* in their tubes from which all crabs had been removed (hosts without conspecifics), and (3) filtered seawater plus two *Chaetopterus* in their tubes with a heterosexual pair of *Pinnixa* in each tube (hosts plus conspecifics). Three trials were run with 15, 15, and 14 different test crabs, respectively, and new hosts were used in each trial. All three reservoirs contained 18 l of seawater at the initiation of the first trial and were aerated for 12 h prior to use. In subsequent trials, the reservoirs contained 21 and 24 l each and were aerated for 14 and 16 h, respectively.

After the initial acclimation period, the pump was turned on (90 min before the first introduction of test crabs) to allow water containing potential host-factor to move through the experimental apparatus. A single *Pinnixa* was then placed in the middle of the central chamber and its position was monitored every 30 min under red light. The crab was allowed to explore the chamber until it entered one of the radial chambers (from which it could not exit), at which time its position was recorded, the crab was removed, and the next crab was placed in the central chamber. Crabs generally entered a radial chamber within 90 min of being placed in the central chamber.

Host by competitor experiment

The design of this experiment was the same as the previous experiment with the exception that the crab pairs residing in the *Chaetopterus* tubes were the competitor *Polyonyx gibbesi* rather than conspecific *Pinnixa*. Initial reservoir volumes were 20 l, and the reservoirs were aerated for 16 h prior to use. All individual test crabs (n = 30) were run sequentially in a single continuous trial.

Hosts only experiments

Only two treatments were used in these experiments: blank seawater controls and hosts in tubes without crabs. Seawater from a given treatment reservoir was pumped into three radial chambers, with the two treatments alternating positions among the six chambers. A single trial was run with two *Chaetopterus* as the hosts and crabs run individually (n = 24 crabs). Reservoir volumes were 18 l, and reservoirs were aerated for 12 h prior to use.

A single trial was also run in which the host tested was *Amphitrite*. Water (40 l) in the treatment reservoir was conditioned with 17 *Amphitrite* (total wet weight 14.7 g) in their tubes for 24 h with aeration before use. Twenty-five crabs were individually tested.

Conspecifics-only experiment

Single, randomly chosen adult *Pinnixa* were placed directly in the radial chambers two hours prior to initiation of a trial. Males and females occupied two radial chambers each, while two chambers were left empty to serve as controls. Chambers containing the same treatment were separated by 180°. Filtered and aerated seawater was pumped into all six radial chambers from a single, periodically refilled reservoir. Single test crabs were placed in the central chamber and monitored as in the other experiments. Three replicate trials (n = 16, 21, and 19 different crabs) were performed.

Table I

Characteristics of the host polychaetes Amphitrite ornata *and* Chaetopterus variopedatus *and their crab symbionts*

	Pinnixa from *Amphitrite*	*Pinnixa* from *Chaetopterus*	*Polyonyx* from *Chaetopterus*
Average wet weight (g)			
male	0.111 (0.050) A	0.477 (0.233) B	0.548 (0.195) B
female	0.125 (0.052) A	0.684 (0.323) C	0.913 (0.376) D
Average length (mm)			
male	3.24 (0.43) A	4.92 (0.93) B	6.30 (0.77) C
female	3.61 (0.58) A	6.15 (1.13) C	7.99 (1.18) D
Average width (mm)			
male	7.34 (1.12) A	10.82 (2.05) C	8.78 (1.10) B
female	7.76 (1.32) A	12.33 (2.52) D	11.46 (1.80) C
Average host weight (g)	0.96 (0.46)	14.32 (4.93)	18.33 (6.54)
Max. diameters of host tubes (cm)	0.5–1.0	1.5–1.75	—
Average volumes of host tubes (ml)	<25.0	44.2 (5.2)	48.0 (8.0)

Amphitrite tube data is taken from Aller and Yingst (1978). Means with the same letter within a row are not significantly different at alpha = 0.05 by one-way ANOVA. Values in parentheses are one standard deviation.

Statistical analysis

The percentages of crabs responding to different treatments in experiments with multiple trials (hosts by conspecifics and conspecifics-only experiments) were compared using three-way analysis of variance with trial, treatment chosen, and sex of the responding crabs as components of the ANOVA. The data were tested for normality and arcsine square-root transformed if necessary. None of the interaction terms were significant so they were dropped from the final analyses. Tukey *a posteriori* tests were performed to compare the mean number of crabs responding to different treatments.

Analyses for all single trial experiments were done using standard Chi-square procedures (Sokal and Rohlf, 1981). In all experiments crabs were tested individually in the choice chamber to eliminate possible behavioral interactions and preserve the assumption of independence of sample responses. Data on crab body sizes in different hosts were analyzed using one-way ANOVA. Statistical analyses were done using SAS, version 6 for PCs (SAS Institute, 1985).

Results

Male and female *Pinnixa* inhabiting *Amphitrite* tubes are significantly smaller than crabs inhabiting *Chaetopterus* (Table I: weight: d.f. = 5, 435, MSE = 0.057, $F = 129.18$, $P < 0.0001$; length: d.f. = 5, 435, MSE = 0.773, $F = 311.94$, $P < 0.0001$; and width: d.f. = 5, 435, MSE = 2.921, $F = 104.85$, $P < 0.0001$). Females of both *Pinnixa* and *Polyonyx* are significantly heavier and have significantly wider and longer carapaces than males of the same species when found in *Chaetopterus*, but female and male dimensions are not significantly different for *Pinnixa* resident in *Amphitrite*. *Polyonyx* females are heavier and longer than either sex of *Pinnixa* in *Chaetopterus*, while males of *Polyonyx* fall within the same size ranges as *Pinnixa*.

These trends in crab body sizes are paralleled by trends in host body sizes. *Pinnixa* resident in *Amphitrite* have hosts with the smallest body weights and smallest tube diameters and volumes, while the *Chaetopterus* hosts of *Polyonyx* have the largest body weights and tube volumes (Table I). Of the 184 *Chaetopterus* hosts examined during the study, 48.9% were occupied by at least one individual of *Polyonyx* (42.9% by a mixed-sex pair), 44.0% by at least one *Pinnixa* (39.7% by a mixed-sex pair), 5.4% by no crab symbionts, and only 1.6% by crabs of both species.

The sizes of male and female *Pinnixa chaetopterana* found together in pairs in *Chaetopterus* hosts from this site are not well correlated. This is true for both $\log_{10}$-transformed body wet weight ($r^2 = 0.079$, $P < 0.028$, $n = 61$) and carapace area ($r^2 = 0.070$, $P < 0.039$, $n = 61$). The correlation of $\log_{10}$-transformed body wet weight ($r^2 = 0.340$, $P < 0.0001$, $n = 67$) and carapace area ($r^2 = 0.123$, $P < 0.004$, $n = 67$) are, however, slightly better for male-female pairs of *Polyonyx gibbesi*.

The number of eggs produced per clutch is highly correlated with female body size in *Pinnixa chaetopterana* ($r^2 = 0.947$, $P < 0.0001$, $n = 27$). The regression equation is:

$$\text{Egg number} = -516 + (44062)(\text{Female dry body weight})$$

Because dry body weight is approximately 31% of average wet weight (Table I), an average-sized female from *Amphitrite* would produce only 1191 eggs per clutch whereas

Table II

Entry choices of Pinnixa chaetopterana *from* Amphitrite *in the* Chaetopterus *host-only experiment*

	Chamber choice		
Sex of test crab	Hosts only	Control	*P*-value
Male	7	7	1.000
Female	6	4	0.527
Total	13	11	0.683

Treatments were unconditioned control seawater and seawater conditioned with two *Chaetopterus* in tubes without any crab symbionts. *P*-values are from Chi-square tests with 1 d.f.

an average *Chaetopterus*-resident female would produce 8826 eggs per clutch.

The results of the hosts-only experiments indicate that neither male nor female *Pinnixa* are attracted to odors emanating from *Amphitrite* or *Chaetopterus* in tubes without crabs (Tables II, III). The control and unoccupied *Chaetopterus* host treatments were therefore combined into a single category for statistical analysis in the host + competitor experiment.

Results of the host + conspecific experiment show that male and female *Pinnixa* respond to odors from conspecifics resident in host tubes (Fig. 2). Crabs of both sexes significantly preferred the treatments in which conspecifics were present with the host. Crabs choosing this treatment were not significantly different in size from those choosing either of the other two treatments (ANOVA: d.f. = 2,38, MSE = 0.002, $F = 0.92$, $P = 0.41$). Neither sex was significantly attracted to the hosts-alone treatment as compared to the control treatment (Fig. 2).

The host + competitor experiment suggests that male and female *Pinnixa* respond somewhat differently to heterospecific odor cues (Table IV). Male *Pinnixa* showed no discrimination between the three treatments, while females show a strong tendency to avoid chambers containing the odor from hosts and *Polyonyx* but do not discriminate between the host odor and control chambers.

Table III

Entry choices of Pinnixa chaetopterana *from* Amphitrite *in the* Amphitrite *host-only experiment*

	Chamber choice		
Sex of test crab	Hosts only	Control	*P*-value
Male	5	10	0.197
Female	3	7	0.206
Total	8	17	0.072

Treatments were unconditioned control seawater and seawater conditioned with *Amphitrite* in tubes without any crab symbionts. *P*-values are from 1 d.f. Chi-square tests.

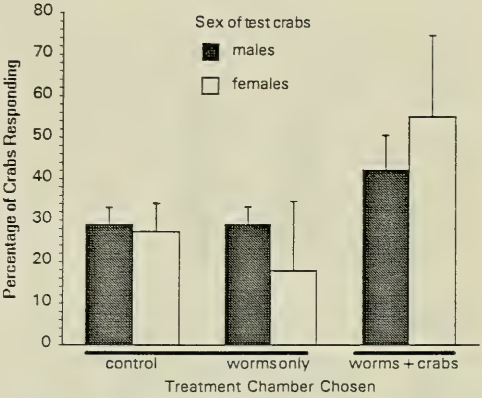

Figure 2. Percentages of *Pinnixa chaetopterana* responding to given treatments in the host + conspecifics experiment. Data are the averages of three trials. Error bars are one standard deviation. Treatments underlined by the same bar are not significantly different at alpha = 0.05.

No sex-based difference in chemosensory responses is evident when conspecific crabs are present but hosts are not (Fig. 3). Male and female *Pinnixa* showed nearly identical distributions among the treatment chambers and were significantly attracted to both sexes.

Discussion

The apparent lack of attraction of adult *Pinnixa chaetopterana* inhabiting *Amphitrite ornata* to odor cues from either *Amphitrite ornata* (Table III) or *Chaetopterus variopedatus* (Table II) parallels the same lack found by Davenport *et al.* (1960) in adult crabs taken from *Chaetopterus*. This result is surprising given that a number of pea

Table IV

Entry choices of Pinnixa chaetopterana *from* Amphitrite *in the host + competitor experiment*

	Chamber choice			
Sex of test crab	Hosts + crabs	Hosts only	Control	*P*-value
Male	4	5	4	0.843
Female	2	8	7	0.059

Treatments were unconditioned seawater, seawater conditioned with two *Chaetopterus* in tubes occupied by heterosexual pairs of *Polyonyx*, and seawater conditioned by two *Chaetopterus* in tubes unoccupied by any crab symbionts. *P*-values are from Chi-square tests with the host-only and control treatments combined to yield 1 d.f.

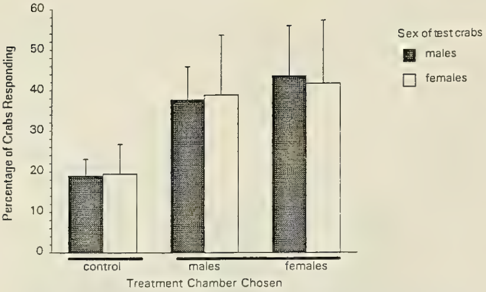

Figure 3. Percentages of *Pinnixa chaetopterana* responding to given treatments in the conspecifics-only experiment. Data are the averages of three trials. Error bars are one standard deviation. Treatments underlined by the same bar are not significantly different at alpha = 0.05.

crab species demonstrate clear attraction to their hosts (Sastry and Menzel, 1963; Gray *et al.,* 1968; Stevens, 1990), with attraction to water-borne cues from previously used hosts found even when the range of potential hosts is quite large (Derby and Atema, 1980). In this experiment, *Pinnixa* individuals actually appear to show a tendency to avoid unoccupied *Amphitrite* hosts (Table III). It may be that these worms were stressed by handling prior to use and were producing unusual odor cues that acted to repel the crabs.

Odor cues from conspecifics could provide wandering crabs with a reliable means of identifying suitable new hosts. This is especially true for symbiotic species such as *Pinnixa chaetopterana* with a large number of potential host species (Williams, 1984). At least one pea crab species with a range of hosts does not respond strongly to initial contacts with odor cues produced by novel host species (Derby and Atema, 1980). *P. chaetopterana* does not respond to odor cues from either previously occupied or new hosts (Tables II, III; Davenport *et al.,* 1960), but does respond positively to conspecific cues with or without the host (Figs. 2, 3).

Attraction to conspecific cues would increase mating opportunities for *Pinnixa.* Hosts may be reliable meeting sites for mating in symbiotic species (Shuster, 1989), and mating often appears to be limited to within the host species (Duffy, 1992; Kearn *et al.,* 1993). In some cases, however, conspecific cues alone are not sufficient for the reliable location of mates and synergism with host-produced odor cues is required (McAuslane *et al.,* 1990; Blumberg *et al.,* 1993). This may be especially important when host quality is correlated with the mating success of symbiotes (Papaj, 1994; Wang and Greenfield, 1994). Synergism between symbiont- and host-produced cues does not appear to be present in this system. The host cue, if present, is incapable of inducing attraction by itself (Tables II, III; Fig. 2) and does not appear to have any synergistic effect, whereas conspecific cues alone are sufficient for attracting crabs to hosts (Fig. 3). It is possible that this particular species requires a tactile cue for positive identification and acceptance of an unoccupied host, as has been found for spider mites (Gotoh *et al.,* 1993).

It is surprising that both male and female crabs are equally attracted to odor cues from both sexes given that there is often strong aggression between members of the same sex in other symbiotic crustacean species (Vannini, 1985; Huber, 1987). However, because 90.1% of *Chaetopterus* hosts occupied by conspecifics already contain a mixed-sex pair (n = 81, this study), a wandering crab is unlikely to find a new host occupied only by a crab of the opposite sex. *Pinnixa* may thus be responding to a generalized odor cue produced by both male and female conspecifics. Odor cues would probably not convey any information as to the size of crabs already present in a host tube, and a crab that enters a tube containing a larger, competitively dominant individual of the same sex would likely move again.

The ability to switch hosts would be adaptive for *Pinnixa chaetopterana* because the crabs are smaller when resident in *Amphitrite* tubes than when in *Chaetopterus* (Table I) and females have reduced reproductive output. Although the potential fitness cost of decreased reproductive capacity due to small size is greater for females than for males in many free-living crabs (Hines, 1982), a male *Pinnixa* that remains in an *Amphitrite* tube will mate with a female with decreased reproductive potential and share the same fitness cost; thus, both sexes would be expected to move to larger hosts after attaining a certain size. Male movement may result in pair formation with a larger female with greater reproductive potential (Knowlton, 1980), but the size-assortative mating found in some symbiotic crustaceans (Adams *et al.,* 1985; but see Diesel, 1988) does not appear to be present in *P. chaetopterana;* the size correlation between mated individuals has an r^2 of less than 0.1.

The potential reproductive cost of small size to female *Pinnixa chaetopterana* in *Amphitrite* may be offset by tradeoffs in the size *versus* number of eggs per brood (Hines, 1982, 1991), but these crabs do not appear to have the reduced body calcification (allowing greatly increased brood mass to body mass ratios) found in other pinnotherids (Hines, 1992). It may also be somewhat offset by a younger age of first reproduction in *Amphitrite*-resident females.

We currently have no explanation as to why female *Pinnixa* tend to avoid *Chaetopterus* hosts that already contain *Polyonyx gibbesi* while male *Pinnixa* do not (Table IV), although asymmetries in the chemosensory and host-location abilities of males and females exist in symbiotic species (Derby and Atema, 1980; Light *et al.,* 1992;

Gotoh *et al.,* 1993). *Polyonyx* is generally found in association with larger *Chaetopterus* than *Pinnixa* (Table I) and may competitively displace the comparatively smaller adult pea crabs resident in smaller tubes as the host worm grows. While male *Pinnixa* have relatively larger claws than females (Williams, 1984), they are unlikely to be dominant over the much larger females of *Polyonyx.* Host avoidance behavior is common in symbiotic species that compete with heterospecifics for control of host resources, especially among parasitic species (McBrien and Mackauer, 1991; Sousa, 1992).

It may be that *Pinnixa chaetopterana* individuals are attracted to host odors as settling larvae or megalopae but subsequently lose this ability with metamorphosis to the adult form. The development of chemotactic responses to conspecific odors would thus be an adaptive response to this rather unusual loss of host attraction. Because 95% of potential *Chaetopterus* hosts are already occupied by either conspecifics or competitors, use of these odor cues may actually be a more reliable estimator of host quality than host odors alone.

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Echinoid Bioerosion as a Major Structuring Force of Red Sea Coral Reefs

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Abstract. Echinoid bioerosion is ecologically important as a limiting factor for reef growth. *Diadema setosum* and *Echinometra mathaei* are the most abundant sea urchins in the Gulf of Eilat, Red Sea. Bioerosion by these urchins was estimated from field experiments with urchins placed in underwater chambers. A novel methodology was developed for measuring the $CaCO_3$ content of fecal pellets and total gut contents. This method is based on measurement of the amount of gas formed by total acidification of a mixed sample. The population density of the urchins was determined for the reef flat (depth 0.6–1 m) and the reef slope (depth 6–8 m). *D. setosum,* which erodes 310 mg individual^{-1} d^{-1}, is much less abundant on the reef flat (0.1 individuals m^{-2}) than on the reef slope (6.4 individuals m^{-2}). In contrast, *E. mathaei,* which erodes 120 mg individual^{-1} d^{-1}, is more dominant on the reef flat (10.5 individuals m^{-2}) than on the reef slope (3.7 individuals m^{-2}). We estimate that echinoid bioerosion converts to carbonate sediments 7%–11% of the total reef flat calcification and 13%–22% of the total reef slope calcification. These findings emphasize the importance of echinoid bioerosion as a structuring force in the coral reef, and its potential importance to the dynamics of reef development. However, they imply that some previous studies may have overestimated the role of echinoid bioerosion, thus possibly underestimating internal bioerosion.

Introduction

The hardness of the teeth of echinoids gives them the ability to scrape or bore into hard carbonate substrates and to ingest calcareous plants (Klinger and Lawrence, 1985). Sea urchins were reported to be very important hard-substrate bioeroders in coral reefs (*e.g.,* Hunter, 1977; Glynn *et al.,* 1979; Trudgill *et al.,* 1987; Bak, 1990), scraping the surface in the process of grazing (Lawrence and Sammarco, 1982). Echinoid bioerosion is considered ecologically important as a limiting factor for reef growth (Glynn *et al.,* 1979), and also has a profound effect on community composition through the control of newly settled coral spat (Sammarco, 1980, 1982).

Reported rates of bioerosion by echinoids are usually in the range of 3–9 kg m^{-2} y^{-1}, depending on the species, intensity of grazing, and population density. Stearn and Scoffin (1977) report a rate of about 9 kg m^{-2} y^{-1} for *Diadema antillarum* in the Caribbean. However, Hunter (1977), who recorded almost the same rate (9.7 kg m^{-2} y^{-1}) for the same species at the same locality, claims that only about 60% of the amount of $CaCO_3$ found in the digestive system of the urchins represents the transformation of hard substrate to sediment, the rest being reworked sediment. Many authors stress the importance of sediment production by echinoids. Glynn *et al.* (1979) found that almost the entire gut content of sea urchins in the Galapagos was composed of particulate carbonate: 30%–40% (weight) was coral skeleton grains; 40%–50% was coralline algae fragments. Hoskin and Reed (1984) measured sediment production of more than 3 kg m^{-2} y^{-1} by *Echinometra lucunter* on Little Bahama Bank.

Among the sea urchins in the northern Gulf of Eilat, *Diadema setosum* and *Echinometra mathaei* are the most abundant (Benayahu and Loya, 1977). Available data on the rate of bioerosion by *E. mathaei* imply a maximum rate of 0.008 kg m^{-2} y^{-1} in Enewetak (Russo, 1980) and 0.4 kg m^{-2} y^{-1} in Moorea (Bak, 1990). The population density of this species in Eilat is similar to that in Moorea. In the northern Gulf of Eilat, bioerosion by echinoids is therefore expected to play a major role in the destruction

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of the reef framework and its conversion to carbonate sediments.

This study aims at (1) quantifying the bioerosion activity of sea urchins in Eilat coral reefs and (2) comparing the results with known rates of reef construction in this area (Barnes and Lazar, 1993).

Materials and Methods

Sea urchins studied in the experiments (both *Diadema setosum* and *Echinometra mathaei*) were collected by scuba diving at the Nature Reserve Reef (NRR), Eilat, Red Sea, at a depth of 4–6 m. Collection was at dawn, when the urchins have completed their nocturnal grazing activity. Seven to ten urchins were put into each of the experimental acrylic chambers (Fig. 1), and the first tube was attached for fecal-pellet collection. The chambers were transferred underwater to the area in front of the H. Steinitz Marine Biology Laboratory (MBL), where they were set at a depth of 4 m. Experiments lasted for 10–10.5 h, and the collection tubes were replaced every 2.5–3 h. Tubes were immediately transferred to the laboratory and stored at 4°C until analyzed (2–12 h later).

Prior to analysis, samples were inspected and any urchin spine was removed. The samples were then homogenized and weighed. Subsamples of 50–100 mg (wet weight) from the homogenate were placed in a 30-ml vessel, along with 1 ml H_2O (to facilitate the reaction) and a tiny plastic cup containing 150 μl concentrated HCl. A cover was screwed on the top and the vessel was sealed. The vessel was then tilted slightly so that the acid cup would tip over, spilling the acid. The acid was then mixed with the sample by gently rotating the vessel. A complete acidification of the sample created CO_2 gas. The CO_2 pressure was read by a 4-digit voltmeter connected to the vessel through a pressure sensor and an analog-to-digital signal convertor. The amount of $CaCO_3$ in the sample is proportional to the measured CO_2 pressure. The apparatus was calibrated with dry aragonite prepared from bleached coral skeletons; its linear range was approximately 0–40 mg $CaCO_3$ (corresponding to pressures of about 1–1.3 atmospheres). This method is much easier and faster than the one formerly used (*e.g.*, Russo, 1980; Scoffin *et al.*, 1980; McClanahan and Kurtis, 1991), which involves several rinse-dry cycles of collection filters.

To estimate the net bioerosion of the reef, the $CaCO_3$ content of fecal pellets of the sea urchins was corrected for reworked $CaCO_3$ particles trapped in the algal turf upon which the urchins graze. Reworked carbonate sediments cannot be considered bioerosion of primary hard-reef substrate, because they were recycled by resuspension. A correction factor was estimated from experiments with "control" urchins grazing on epilithic algae that grow on granite-pebble talus and beachrocks, located about 100–300 m south of the NRR. The carbonate particles in this area were transported by either currents or wind and are therefore recycled detrital carbonate. Due to limitations imposed by the Nature Reserves Authority, there is only one control group for each species.

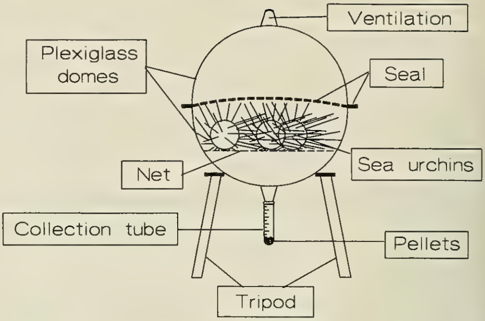

Figure 1. Schematic presentation of the underwater experimental chambers. The urchins were placed on a plastic mesh (*ca.* 1 cm). At each sampling time (every 2.5–3 h) the collection tube was replaced. Following each sampling, the top dome was removed for about 10 s, and gentle currents were created manually to ensure sufficient water replenishment.

Several groups of sea urchins (corresponding to experimental groups kept together in an experimental chamber) were dissected, and total soft tissue was analyzed for $CaCO_3$ content by the method described above. Data were used to calculate the residence time of $CaCO_3$ in the alimentary tract of sea urchins, by dividing the total amount of $CaCO_3$ present at the beginning of the experiment (fecal contents + alimentary tract contents) by the $CaCO_3$ defecation rate. Height and diameter of the test and of Aristotle's lantern were measured for each dissected urchin.

The population density of sea urchins in two distinct reef zones, (1) the reef flat (depth 0.6–1 m) and (2) the reef slope (depth 6–8 m), was determined by belt transects (10×1 m^2). Transect censuses were done at night, after the urchins were fully active, by scuba diving. The total area counted was 80 m^2 for *D. setosum* and 100 m^2 for *E. mathaei*.

Results

Figure 2 shows the percent $CaCO_3$ found in the alimentary tract of *Diadema setosum* collected from three types of substrate: (1) calcareous reef rock, (2) talus of large granite pebbles, and (3) coarse-grain beachrock composed mainly of igneous rock grains with carbonate (high-Mg calcite) cement. The gut contents of urchins from the reef contained about three times more $CaCO_3$ than that of urchins from the granite pebbles and beachrock.

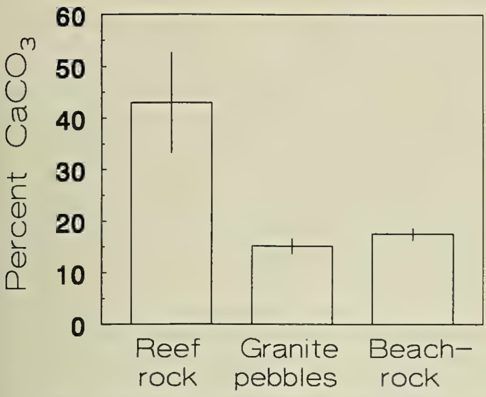

Figure 2. Percent $CaCO_3$ in gut contents of *Diadema setosum* collected from different substrates: calcareous reef rock, granite pebbles, and beachrock. Data represent average ±SD of 10, 5, and 5 urchins, respectively.

The results of feces collection experiments for *D. setosum* and *E. mathaei* are given in Figure 3 and Table I. The $CaCO_3$ defecation rates are the slopes of linear regression lines in Figure 3. Note that lines in the figure do not intersect at the origin. This is due to the exceptionally high amount of $CaCO_3$ collected at the end of the first time interval. The anomalously high defecation rate measured for the first time interval was probably a result of disturbance during the transfer of the experimental chambers from the site of urchin collection to the experimental area, and is therefore considered to be an artifact. The "control" urchins, which were collected on the granite pebbles or the beachrock near the experimental area (thus being less disturbed), showed no change in defecation rate. Consequently, the results of the first time interval were not used for calculating the defecation rate. The average rate of $CaCO_3$ defecation for *D. setosum* is 24.6 ± 8.4 mg individual^{-1} h^{-1} for the experimental groups and 11.7 mg individual^{-1} h^{-1} for the control. $CaCO_3$ defecation rate for *E. mathaei* is 5.3 ± 2.1 mg individual^{-1} h^{-1} for the experimental groups and 0.25 mg individual^{-1} h^{-1} for the control.

Table II summarizes the physical parameters of *D. setosum* and *E. mathaei* and their distribution on the reef. Test diameter and height of *D. setosum* are 1.3 and 1.5 times (respectively) larger than the corresponding parameters of *E. mathaei*. Consequently, the test volume of *D. setosum* is computed to be 2.7 times larger. The distributions of *D. setosum* and *E. mathaei* in the studied reef zones are markedly different. Whereas *D. setosum* is most abundant over the reef slope, *E. mathaei* dominates the reef flat.

Discussion

Each of the experimental groups, in both species studied, defecated $CaCO_3$ at a relatively constant rate (r^2 values of the regression slopes are noted in Fig. 3). Despite the similarity of the slopes, however, the intersects vary widely (especially for *D. setosum*, Fig. 3A). This may be attributed to different "stress reactions" to the transport from the Nature Reserve to the MBL. The duration of the anomalously high defecation rate is probably variable, but seems to range between 0.5 h and 2.5 h. The lines describing $CaCO_3$ defecation of all experimental groups for which the first collection tube was replaced after 2.5 h or more (all *D. setosum* groups and *E. mathaei* groups I and II, Table I) are typified by very high r^2 values (Fig. 3), reflecting constant rates. The three *E. mathaei* groups, for

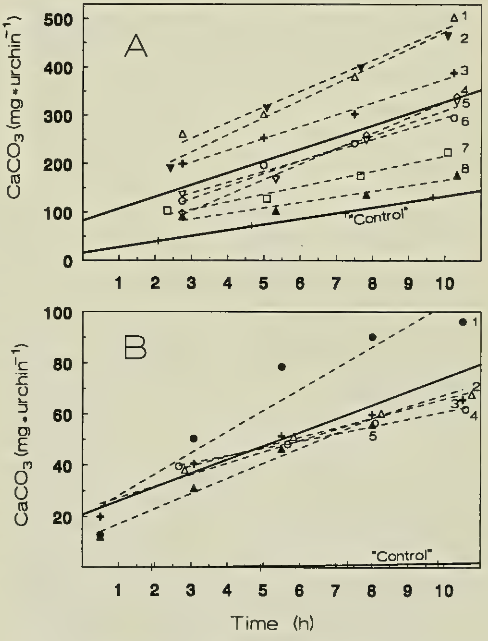

Figure 3. Cumulative amount of $CaCO_3$ in fecal pellets collected from the underwater experimental chambers for (A) *Diadema setosum* and (B) *Echinometra mathaei.* In each part: dashed lines represent each experimental group; top solid line represents data combined from all experimental groups; bottom solid line represents the "control" group. Linear curves were fitted by regression analysis. Slopes and r^2 values for individual lines are as follows. (A): (1) 32.4, 0.94; (2) 35.5, 0.96; (3) 24.7, 0.98; (4) 32.1, 0.98; (5) 26.1, 0.94; (6) 22.4, 0.96; (7) 15.9, 0.94; (8) 11.5, 0.90; (combined data) 24.6, 0.41; ("Control") 11.7; 0.98. (B): (1) 8.3, 0.81; (2) 3.8, 0.98; (3) 4.5, 0.88; (4) 2.9, 0.98; (5) 5.9, 0.96; (combined data) 5.3, 0.69; ("control") 0.25, 0.74. Note that the *y*-axis scale is five times smaller for *E. mathaei* than for *D. setosum.*

Table I

Results of 10-h feces collection experiments (data represent one experimental group except where noted[a])

Species	$CaCO_3$ (mg)		Gut evacuation (%)	Gut turnover rate (d^{-1})
	Defecated	Remaining in gut		
Diadema setosum	296	603	33 (±1)[a]	0.75
Echinometra mathaei	67	38	64	1.5

[a] The SD was calculated together with two additional experimental groups dissected and analyzed (results not shown).

Table II

Physical parameters and population densities of the echinoids Diadema setosum *and* Echinometra mathaei *in the Eilat Nature Reserve reef (values represent the average ± 1 standard deviation)*

Species	Physical parameters (mm)				Population density (urchin m^{-2})[a]	
	Test		Aristotle's lantern			
	Diameter	Height	Diameter	Height	Reef slope	Reef flat
Diadema setosum	30.1 ± 7.6 $n = 50$	17.3 ± 4.9 $n = 50$	11.2 ± 2.7 $n = 50$	14.8 ± 3.4 $n = 50$	6.4 ± 3.1 (60)	0.1 ± 0.3 (20)
Echinometra mathaei	23.1 ± 4.5 $n = 20$	11.9 ± 2.2 $n = 20$	7.0 ± 1.1 $n = 20$	8.8 ± 1.6 $n = 20$	3.7 ± 3.3 (60)	10.5 ± 5.6 (40)

[a] Numbers in parentheses are the total area (in square meters) censused by belt transects.

Table III

Estimates of echinoid bioerosion rates

Species	Bioerosion (g individual^{-1} d^{-1})	Population density (individual m^{-2})	Locality	Method		Reference
				$CaCO_3$ measurement	Correction for reworked sediments	
Diadema antillarum	1.070	23	Barbados	Acidification of total gut contents (weight difference)	No correction	Stearn and Scoffin, 1977
	1.160	23	Barbados	Unpublished data from C.M. Hawkins	Petrographic sections	Hunter, 1977
	0.630 ± 0.040	23	Barbados	Acidification of total gut contents (weight difference)	Petrographic sections	Scoffin *et al.*, 1980
Diadema savignvi	1.920	4.8	Moorea	Ash-free dry weight of total gut contents	Petrographic sections	Bak, 1990
Diadema setosum	0.310 ± 0.200	Reef flat 0.1 ± 0.3 Reef slope 6.4 ± 3.1	Eilat	Acidification of excreted fecal pellets (CO_2 pressure measurement)	According to urchins from a noncarbonate substrate	This study
Echinometra mathaei	0.140	0.16	Enewetak	Acidification of total gut contents (weight difference)	Based on 20% reworked sediment—unpublished calculations by Peterson	Russo, 1980
	0.120	7.4	Moorea	Ash-free dry weight of total gut contents	No correction	Bak, 1990
	0.120 ± 0.050	Reef flat 10.5 ± 5.6 Reef slope 3.7 ± 3.3	Eilat	Acidification of excreted fecal pellets (CO_2 pressure measurement)	According to urchins from a noncarbonate substrate	This study

which the first collection tube was replaced after 0.5 h, show somewhat lower r^2 values, and the defecation rate declines a little. The implications of this decrease for further calculations are discussed below.

The rate of bioerosion is calculated separately for each zone on the reef, by multiplying the average $CaCO_3$ defecation rate per individual by the population density in the reef-zone of interest. Total urchin bioerosion was calculated to be approximately 0.5 kg $CaCO_3$ m^{-2} y^{-1} on the reef flat and 0.9 kg $CaCO_3$ m^{-2} y^{-1} on the reef slope (Fig. 4). Typical reef accretion (calcification rate) in this area is approximately 4–7 kg $CaCO_3$ m^{-2} y^{-1} (Barnes and Lazar, 1993). The bioerosion activity of sea urchins alone, therefore, counteracts only about 7%-11% of the total calcification on the reef flat, and 13%-22% of that on the reef slope. The effect of echinoid bioerosion in the two reef zones studied is depicted in Figure 4, emphasizing the relative importance of each sea urchin species in each zone.

E. mathaei clearly erodes much less $CaCO_3$ per individual than does *D. setosum* (Fig. 3, Table I). Consequently *E. mathaei* is responsible for only about 18% of the echinoid bioerosion on the reef slope (Fig. 4), although it constitutes nearly 40% of the urchin population there (Table II). On the other hand, it is responsible for over 97% of echinoid bioerosion on the reef flat, where it is much more abundant than *D. setosum* (Table II). The smaller size of *E. mathaei* and its stronger attachment to the substrate probably enable this urchin to dominate the reef flat, which is typified by high-energy wave and current action.

Considerable differences exist between estimates of echinoid bioerosion (Table III). It is, therefore, interesting to compare the methods by which these estimates were obtained. Many authors (*e.g.*, Scoffin *et al.*, 1980; Bak, 1990) have used total $CaCO_3$ gut content of dissected urchins as an estimate for daily erosion. Besides being a rather destructive method (all studied urchins are killed), it may yield erroneous results. Sea urchins of different size classes, or of different species, may have profoundly different gut contents, but their gut turnover rates may vary just as much. Thus, urchins having larger gut contents do not necessarily ingest more food and $CaCO_3$ per unit time. Markedly different gut turnover rates were calculated for the two species studied here (Table I). The faster turnover rate of *E. mathaei* might be accounted for if a smaller space was occupied by the intestine within the test. Although its Aristotle's lantern is relatively smaller (*D. setosum* has a 2.7 times larger test and a 4.7 times larger lantern, see Table II), the absolute volume left for soft tissue is still about 2.7 times smaller in *E. mathaei*. This may limit the ability of the intestine to expand.

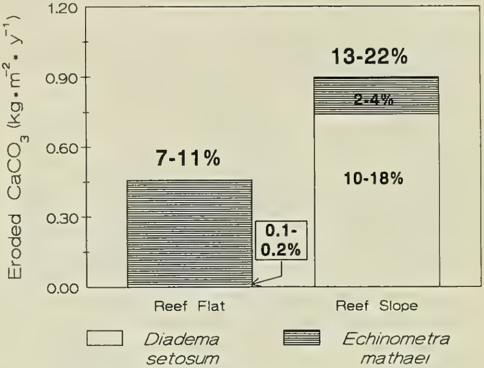

Figure 4. Bioerosion by *Diadema setosum* and *Echinometra mathaei* in two reef zones in Eilat, Red Sea. Open and hatched areas of each bar represent the proportion of reef calcification that is eroded by each urchin species. The total fraction of reef accretion eroded by these echinoids is shown above the bars. Note that urchin bioerosion on the reef flat is almost exclusively due to the activity of *E. mathaei*, whereas most of the urchin bioersion on the reef slope is due to *D. setosum* activity.

Estimation of the rate of $CaCO_3$ ingestion from measurements of $CaCO_3$ in collected fecal pellets is based on the assumption of a gross "steady state" regarding gut contents. Such an assumption is widely accepted (*e.g.*, McLean, 1967; Glynn, 1979; McClanahan and Kurtis, 1991). Whereas fecal-pellet collections record true values for urchins of all sizes and species, dissections may overestimate bioerosion by very large urchins with low gut turnover rates, such as *D. setosum*. This may partially account for Bak's (1990) high estimates for echinoid bioerosion in Moorea: *Diadema savignyi*, with an average test diameter of 54 ± 10 mm (calculated from data presented by Bak), is responsible for about 75% of the bioerosion there.

Knowing the amount of $CaCO_3$ that passes through the digestive system of a sea urchin, one still has to correct for the amount of $CaCO_3$ that is derived from reworked sediments. Kendrick (1991) reports 200–1800 g of sediment (dry weight) trapped in 1 m^2 of algal turf. Uncorrected high estimates of echinoid bioerosion have been published (*e.g.*, Lewis, 1964) with the notion that correction is desirable (Stearn and Scoffin, 1977). Hunter (1977) and Bak (1990) used thin-section microscopy to differentiate between $CaCO_3$ particles directly eroded by the urchin and reworked material. This technique requires considerable work for slide preparation and examination. In our study the correction was more easily obtained by comparing the amount of $CaCO_3$ measured for urchins grazing on reef rock with the amount in urchins grazing on igneous rock and beachrock (see Fig. 2). The proximity of the two sites and the prevailing current regime result in transportation of suspended particles between these sites, exposing "reef" and "control" urchins to similar amounts of reworked $CaCO_3$.

An important part of our experimental design is the timing and duration of the experiments. To avoid interruption of the urchins' dial biological rhythms, all experiments were carried out during the day, when the urchins are inactive. Calculation of daily bioerosion was based on the assumption that defecation rate is constant throughout day and night. This condition is probably well met by the urchins, which show very constant defecation rates throughout the experiments (Fig. 3), despite the gradual emptying of the gut. The substantial amount of $CaCO_3$ remaining in the gut at the end of the experiment (Table I) also supports the assumption of a constant rate of defecation. These observations contradict a suggestion made by Lawrence and Hughes-Games (1972) that these nocturnally feeding echinoids might have higher defecation rates at night. However, even if defecation were twice as fast during nighttime feeding, our estimated rate of bioerosion (Fig. 4) would not change much. In such a case, bioerosion by echinoids would be about 0.6 kg m^{-2} y^{-1} on the reef flat (9%–15% of reef accretion) and about 1.2 kg m^{-2} y^{-1} on the reef slope (17%–29% of reef accretion).

Evidently, the overall effect of echinoid bioerosion on the reef framework in the study area is of considerable importance. Our results emphasize the already acknowledged importance of bioerosion as a structuring force in coral reef areas. However, they imply that some previous studies may have overestimated the impact of echinoid bioerosion. Concurrently, the role played by other erosion forces, including internal bioerosion and physical erosion (mainly on the reef flat), was possibly underestimated. Erosion by agents other than echinoids probably plays a central role in the area studied in the present research, as indicated by a 70%–90% discrepancy between reef accretion and echinoid bioerosion. Our conclusions thus call for follow-up investigations to evaluate the relative importance of other major reef bioeroders in the Red Sea. Such studies should provide important complementary information on the overall importance of bioerosion in this area.

Acknowledgments

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Inorganic N Assimilation and Ammonium Pools in a Deep-Sea Mussel Containing Methanotrophic Endosymbionts

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Abstract. Undescribed mussels (seep mytilid la) harboring methanotrophic endosymbionts exhibit high biomass around hydrocarbon seeps on the Louisiana Slope of the Gulf of Mexico. These mussels assimilate ammonium and nitrate present at high concentrations in their environment. Pathways of assimilation were investigated by enzyme activity measurements and ^{15}N tracer experiments. Glutamine synthetase was detected in all freshly collected mussels tested. Nitrate reductase activity was not always observed. Exposure to $^{15}NH_3$ resulted in the appearance of millimolar concentrations of $^{15}NH_3$ within the symbiont-containing tissues. The concentration of internal $^{15}NH_3$ was several times higher than in the medium and correlated with $^{15}NH_3$ assimilation rate. These results indicate that exogenous $^{15}NH_3$ was taken up into a large internal pool before it was assimilated. Our results do not indicate the extent to which ammonium pools were within the host or symbiont or whether ammonium assimilation was facilitated by either partner exclusively. The observation of elevated internal ammonium concentrations is inconsistent with the "depletion–diffusion" mechanism of nutrient uptake proposed for algal–invertebrate symbioses and is suggestive of active ammonium uptake mechanisms across the host surface. Exposure to $^{15}NO_3$- also resulted in the appearance of $^{15}NH_3$, with internal $^{15}NH_3$ concentration correlated with $^{15}NO_3$- assimilation rate. This result indicates that $^{15}NO_3$- was reduced more rapidly than it was assimilated and that $^{15}NH_3$ derived from $^{15}NO_3$- may also enter an internal ammonium pool. Assimilation of nitrate in the presence of millimolar concentrations of internal ammonium and reduction of $^{15}NO_3$- in excess of assimilation is consistent with the functioning of dissimilatory nitrate reduction pathways with ammonium as a major endproduct. Such a mechanism may operate in other chemosynthetic symbioses that exhibit dissimilatory nitrate reduction.

Introduction

Symbiotic associations between chemosynthetic bacteria and marine invertebrates are well documented in areas where reduced chemical species are present; these areas include hydrothermal vents, cold seeps, sewage sludge outfalls, seagrass beds, and anoxic basins (Fisher, 1990). Undescribed deep-sea mussels (seep mytilid la) that harbor endosymbiotic methanotrophic bacteria in their gill filaments (Childress *et al.*, 1986; Fisher *et al.*, 1987) are found around hydrocarbon seeps on the continental slope of the Gulf of Mexico (MacDonald *et al.*, 1989). Although these mussels filter-feed (Page *et al.*, 1990), symbiont methanotrophy is an important energy and carbon source. Seep mytilids assimilate methane at high rates (Childress *et al.*, 1986; Kochevar *et al.*, 1992), exhibit shell growth in the presence of methane as the sole carbon source (Cary *et al.*, 1988), and have highly depleted $\delta^{13}C$ values that are similar to those of methane in the environment (Brooks *et al.*, 1987; Kennicutt *et al.*, 1992). In terms of the acquisition of elemental constituents, physiologists studying chemosynthetic symbioses have focused their attention primarily on sources and assimilation pathways of carbon. Because nitrogen is an important and often limiting elemental constituent of organisms, we have recently endeavored to delineate the sources of ni-

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trogen to these associations and the mechanisms involved in nitrogen uptake and assimilation.

Biomass at seep sites is high, ranging up to >37.7 kg m^{-2} (C. R. Fisher, pers. comm.), which may in part be the result of the high availability of inorganic nitrogen. Seep mytilids encounter rich sources of ammonium and nitrate. The porewater of sediment near mussels and hypersaline seep effluent contains millimolar concentrations of ammonium (MacDonald *et al.,* 1990a; Lee and Childress, 1994). Nitrate in bottom water is about 40 μM (Lee and Childress, 1994). In contrast, symbiotic associations between algae and invertebrates encounter considerably lower concentrations—*e.g.,* 0.2–1.4 μM ammonium; 0.2–1.52 μM nitrate (Muscatine, 1980). Elevated source concentrations of nitrogen may also explain why seep mytilids exhibit depleted $\delta^{15}N$ values compared with organisms found elsewhere in the marine environment (Brooks *et al.,* 1987; Kennicutt *et al.,* 1992), since such conditions could result in a high degree of ^{15}N discrimination during uptake and assimilation.

We have documented that these and other chemosynthetic symbioses can assimilate inorganic N. Seep mytilids take up and assimilate ammonium and nitrate but not N_2 (Lee *et al.,* 1992b; Lee and Childress, 1994). In ^{15}N tracer studies, most of the label appears in the symbiont-containing gill tissue (Lee and Childress, 1994) as low molecular weight compounds (Lee and Childress, 1995). Comparison of rates of ammonium and nitrate uptake and assimilation with rates of methane assimilation indicates that these sources can contribute significantly toward the nitrogen needs of the association (Lee *et al.,* 1992b; Lee and Childress, 1994, 1995). Although the capacity to utilize inorganic nitrogen is of importance to these and other chemosynthetic bacteria–invertebrate symbioses, little is known about mechanisms and pathways of assimilation. In the present study, we used ^{15}N-labeled ammonium and nitrate to investigate assimilation by the seep mytilid in experiments involving intact mussels and excised gills. Concentrations of $^{15}NH_3$ (the sum of ionized and nonionized forms) within tissues and the relation between assimilation and $^{15}NH_3$ concentration were determined. Activities of nitrogen-assimilating enzymes, nitrate reductase, glutamine synthetase, and glutamate dehydrogenase were measured.

Materials and Methods

Collection and maintenance of mussels

Mussels were collected in August 1991, August and September 1992, and June and July 1993 on the Louisiana Slope of the Gulf of Mexico (27°N; 91°W) by the DSRV *Sea Link I* and *II* and maintained in the laboratory as described previously (Lee *et al.,* 1992b) at 5°C (similar to the temperature at the collection sites) in nonpressurized seawater aquaria. The effect of the pressure difference between the collection sites (540–650 m) and the conditions in the laboratory and during experiments was apparently negligible, because the mussels and symbionts can persist for more than a year in captivity. In 1991, mussels were collected from Bush Hill (MacDonald *et al.,* 1989). The 1992 mussels were collected at Bush Hill and the Brine Pool (MacDonald *et al.,* 1990b), but were not kept separated, whereas the 1993 mussels were from either Bush Hill, the Brine Pool, or GC-272 (Brooks *et al.,* 1989a).

Assimilation experiments with whole mussels

Mussels were incubated at 5°–7°C in the presence of methane and $^{15}NH_3$ or $^{15}NO_3$- for 6–24 h. Concentrations of ammonium and nitrate in the incubations were determined by flow-injection analysis (FIA; Johnson and Petty, 1983; Willason and Johnson, 1986). Methane, N_2, and oxygen were analyzed by gas chromatography (GC; Childress *et al.,* 1984). Concentrations of N substrates and methane were maintained by regular additions of concentrated stock solutions. Methane stock solutions ($\approx$800–900 μM) were made by equilibrating methane with seawater. At the end of each incubation, mussels were dissected into gill (symbiont-containing) and mantle (symbiont-free) tissues, then blotted on a paper towel and immediately dried at 60°C.

Assimilation experiments with excised gills

Gills with a small portion of mantle tissue left attached were placed in petri dishes containing 0.2-μm filtered seawater (FSW) at 5°C, then subdivided by cutting parallel to the filaments. These gill pieces were placed in syringes or plastic bottles (250–1000 ml) containing ^{15}N and ^{13}C substrates. Air bubbles were removed and the syringes or bottles placed on a shaker table at 5°C for 0.25–24 h. At the end of the incubations, mantle tissue was removed and the remainder blotted, then dried at 60°C.

Preparation of samples and determination of $^{15}N/^{14}N$

Ammonium was removed from seep mytilid tissue by adding 100–200 μl 2*N* NaOH to samples that had been dried and ground to a fine powder. This converted NH_{4^+} to NH_3 gas which was then lost to the atmosphere. These treated samples were kept at room temperature for more than 24 h before they were redried and analyzed for $^{15}N/^{14}N$. This treatment consistently resulted in quantitative loss of $^{15}NH_3$ from the samples (see Results). Removal of unassimilated $^{15}NH_3$ was necessary to determine assimilation into organic compounds. Untreated samples were also analyzed, and the $^{15}NH_3$ lost was quantified by difference and taken to be a measure of internal $^{15}NH_3$.

$^{15}N/^{14}N$ and %N present in both the NaOH treated and untreated samples were determined using an automated

CHN analyzer (Roboprep-CN; Europa Scientific) interfaced with an isotope ratio mass spectrometer (Tracermass, Europa Scientific) as described by Owens (1988); Preston and McMillan (1988); Brooks *et al.* (1989a); Owens and Rees (1989). The procedure was as follows. Dried samples were ground to a fine powder, and 1 to 2 mg of sample was placed in a tin capsule and weighed to ±0.001 mg with a Cahn electrobalance. The encapsulated samples were flash-combusted at 900°C and reduced over copper metal at 550°C. The N_2 and CO_2 produced were separated by GC, then admitted directly to the isotope ratio mass spectrometer for determination of $^{15}N/^{14}N$ as well as $^{13}C/^{12}C$. We routinely obtained precision of better than 0.01 atom% ($^{15}N \times (\text{total N})^{-1} \times 100$) for ^{15}N and ±0.1% for %N using reference materials (NBS 1572, citrus leaves).

Enzyme assays

Mussels were dissected on ice and tissues were homogenized, with a Polytron tissue homogenizer at 10,000 rpm, for 2 min in 3–5 volumes of 50 m*M* Tris buffer at 5°C and pH 7.0.

Nitrate reductase was assayed in a reaction mixture containing 175 m*M* phosphate, 5 m*M* sodium nitrate, 200 μM benzyl viologen, and 2.3 m*M* sodium dithionite at pH 7.4 and 20°–25°C. Appearance of nitrite was followed by addition of sulfanilamide and α-napthylethylenediamine to form an azo-dye that was measured spectrophotometrically at 543 nm.

Activities of ammonium assimilation enzymes, which could be of both host or symbionts, were determined as follows. Glutamine synthetase was assayed by measuring its transferase activity according to the methods of Pregnall *et al.* (1987) in a reaction mixture containing 200 m*M* Tris, 3 m*M* $MnCl_2$, 20 m*M* sodium arsenate, 50 m*M* L-glutamine, 4 m*M* ADP, and 150 m*M* hydroxylamine at pH 7.3 and 25°C. The reaction product, γ-glutamyl hydroxamate, was measured following addition of a mixture of $FeCl_3$, TCA, and HCl to stop the reaction and form iron-γ-glutamyl hydroxamate, which was then analyzed spectrophotometrically at 543 nm. Glutamate dehydrogenase was assayed in a reaction mixture containing 50 m*M* Tris, 5 m*M* α-ketoglutarate, and 0.25 m*M* NAD(P)H at pH 7.5 and 25°C. The reaction was initiated by addition of ammonium to a final concentration of 50 m*M*. The rate of NAD(P)H oxidation was determined spectrophotometrically at 340 nm. Protein was determined in tissue homogenates by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Removal of $^{15}NH_3$ and determination of internal $^{15}NH_3$ concentration

To assess whether $^{15}NH_3$ was specifically and quantitatively removed by treatment with 2*N* NaOH, control experiments were conducted using homogenized tissue from seep mytilids not exposed to ^{15}N-labeled substrates. A series of samples ($^{15}NH_3$-enriched samples) containing varying amounts of ^{15}N was generated by addition of $^{15}NH_4Cl$ solution (untreated, Fig. 1a). After NaOH treatment, ^{15}N enrichment due to $^{15}NH_4Cl$ was lost, with atom % ^{15}N values only slightly elevated compared to samples with no $^{15}NH_3$ enrichment (Fig. 1a). These data indicate that NaOH treatment resulted in virtually complete removal of ammonium. To test whether ^{15}N from amino acids was volatilized during processing and NaOH treatment, a similar experiment was conducted by enriching seep mytilid tissue with varying amounts of ^{15}N-glycine. Loss of ^{15}N following treatment with NaOH was not detected (Fig. 1b). These results indicated that the NaOH treatment was quantitative and specific for ammonium.

In this paper, "assimilation" of $^{15}NH_3$ refers to ^{15}N incorporation into the NaOH-treated fraction and represents incorporation of $^{15}NH_3$ into organic compounds. From changes in atom % ^{15}N resulting from treatment

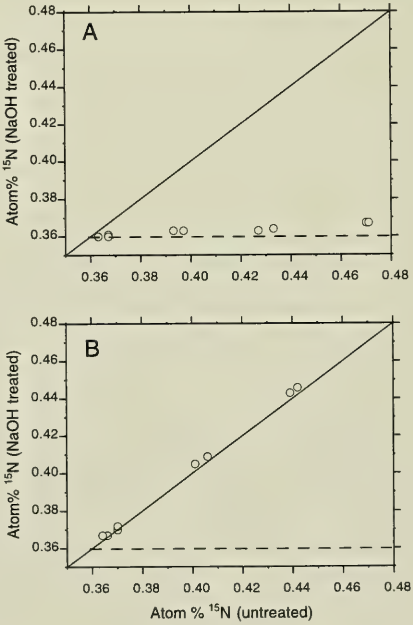

Figure 1. Atom% ^{15}N of seep mussel samples before and after treatment with NaOH for (A) $^{15}NH_4Cl$ and (B) ^{15}N-glycine enriched samples. Dashed line (based on unenriched samples = 0.36 atom %) is the relationship predicted if removal of added ^{15}N is quantitative. Solid line (unity) is the relationship predicted if NaOH treatment does not result in loss of ^{15}N.

with NaOH and %N results, we also calculated moles of $^{15}NH_3$ lost per sample. Internal $^{15}NH_3$ (m*M*) will refer to calculated values of mmoles ^{15}N lost per kilogram water present in tissue. Since $^{14}NH_3$ was probably also present, internal $^{15}NH_3$ concentrations were less than actual internal ammonium ($^{15}NH_3$ and $^{14}NH_3$) concentrations. Gill-tissue $^{15}NH_3$ values reflect both symbiont and host ammonium pools. Most of the gill is host tissue. Although there are no published estimates of symbiont volume in seep mytilid gills, electron micrographs of cross-sections of gill filament cell from Kochevar *et al.* (1992) and Fisher *et al.* (1987) are 11% and 14% symbionts respectively.

$^{15}NO_3$- assimilation rates

In this study, $^{15}NO_3$- "assimilation" will refer to incorporation of $^{15}NO_3$- observed in NaOH-treated samples. Although treatment of samples with NaOH removed $^{15}NH_3$, unassimilated $^{15}NO_3$- should not have been volatilized. Consequently, these samples probably contained $^{15}NO_3$- as well as ^{15}N in organic compounds. There was no compelling reason to believe that much of the ^{15}N was in the form of $^{15}NO_3$-. When substantial $^{15}NO_3$- incorporation was observed, it was not the result of simple equilibration of internal and external [$^{15}NO_3$-]. In two experiments in which moderate rates of $^{15}NO_3$- assimilation were exhibited, the concentration of incorporated ^{15}N was considerably greater than the concentration of $^{15}NO_3$- in the medium at external $^{15}NO_3$- less than 1 m*M* (Fig. 2). This ^{15}N was primarily in the form of organic compounds unless the host or symbiont exhibited a substantial capacity for nitrate sequestration. In an experiment in which excised gills exhibited negligible $^{15}NO_3$- assimilation, the concentration of incorporated ^{15}N was clearly less than the concentration of $^{15}NO_3$- in the medium (Fig. 2).

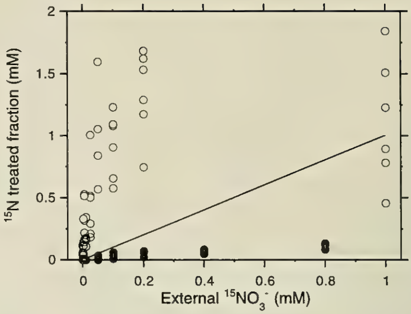

Figure 2. Comparison of concentration of ^{15}N incorporated with concentration of $^{15}NO_3$- in the medium. Open circles = excised gills (1992 collection, 6 individuals, 3- or 6-h incubation); filled circles = excised gills (1992 collection, 3 individuals, 3-h incubation), negligible assimilation. Each data point represents a single gill-piece incubation. Solid line = unity.

Assimilation of $^{15}NH_3$ and $^{15}NO_3$- by seep mussels

Symbiotic seep mussels generally exhibited a higher capacity for assimilation of ammonium than for assimilation of nitrate. Ammonium assimilation by symbiotic mussels and excised gills was always observed and at fairly consistent rates, whereas nitrate assimilation was not always observed and assimilation rates varied greatly (Table I). It should be noted that $^{15}NH_3$ assimilation could have been facilitated by either host or symbiont. Assimilated ^{15}N from $^{15}NH_3$ and $^{15}NO_3$- incubations involving whole mussels was found primarily in the gill tissue (Table I). As a result, discussion of our results from whole-mussel experiments will be limited primarily to ^{15}N incorporation into gill tissue. Previously, we reported that rates of $^{15}NO_3$- assimilation into excised gills and gills of whole mussels are one-sixth to one-fourth the rates exhibited for $^{15}NH_3$ assimilation (Lee and Childress, 1994, 1995). In the present study we found that higher rates of $^{15}NO_3$- assimilation can occur. Mussels collected in 1992 exhibited rates of $^{15}NO_3$- assimilation similar to those observed in 1991 (Table I). Excised gills from mussels collected in 1992 had rates of $^{15}NO_3$- assimilation higher than those found with whole mussels (Table I). Similar rates of $^{15}NO_3$- and $^{15}NH_3$ assimilation were exhibited by excised gills of mussels from the 1992 collection incubated in the presence of 50 μM ^{15}N-labeled substrate (Table I). Not all excised gills exhibited $^{15}NO_3$- assimilation. Two other collections of mussels arrived at the laboratory in 1992. Excised gills of mussels from one had lower rates of $^{15}NO_3$- assimilation (0.106 ± 0.014 μmol g^{-1} h^{-1} (SD, $n = 3$) in the presence of 50 μM $^{15}NO_3$-). Negligible rates of $^{15}NO_3$- assimilation were observed in the other (0.003 ± 0.003 μmol g^{-1} h^{-1} (SD, $n = 3$) in the presence of 50 μM $^{15}NO_3$-).

The highest rates of $^{15}NO_3$- assimilation by whole mussels were observed in 1993 shipboard experiments. Mussels from two sites, Bush Hill and Brine Pool NR-1, were used less than 48 h after collection. Moderate rates of $^{15}NO_3$- assimilation into gill tissue were observed for mussels from both sites. In paired experiments, rates of ^{15}N assimilation were higher with $^{15}NH_3$ as N source. Mussels collected at Brine Pool NR-1 and incubated in $^{15}NO_3$- exhibited high rates of assimilation less than 3 d after their arrival in the laboratory. Mussels from the same collection exhibited substantially lower rates of $^{15}NO_3$- assimilation after 1 month of maintenance in the laboratory (Table I). The finding that $^{15}NO_3$- assimilation was negligible in whole-mussel incubations from 1991 and 1992 but that rates were moderate in 1993 may be a func-

Table I

Rates of assimilation of $^{15}NH_3$ and $^{15}NO_3$- into gill tissue of whole seep mussels incubated on board ship or after maintenance in the laboratory; incubations were 7–18 h in the presence of 100 μM ^{15}N substrate and 200 μM methane

		^{15}N assimilation (μmol g^{-1} h^{-1})	
Site/conditions	Tissue	$^{15}NO_3$	$^{15}NH_4$
Unspecified location, 1991 collection*			
Laboratory maintained (<7 d)	Gill	0.061 ± 0.047 (17)	0.361 ± 0.161 (16)
	Mantle	0.004 ± 0.002 (10)	0.038 ± 0.022 (10)
Unspecified location, 1992 collection			
	Gill	0.059 ± 0.031 (5)	0.314 ± 0.145 (12)
	Mantle	0.013 ± 0.009 (5)	0.016 ± 0.013 (12)
	Excised gill	0.266 ± 0.070 (5)	0.280 ± 0.106 (7)
Bush Hill, 1993 collection			
Freshly collected	Gill	0.310	0.210
		0.095	0.173
		0.099	0.253
	Mantle	0.005	0.008
		0.001	0.007
		0.002	0.009
Brine Pool, 1993 collection			
Freshly collected	Gill	0.087	0.283
		0.086	0.523
		0.102	0.633
	Mantle	not detected	0.003
			0.001
			0.002
Laboratory maintained (3 d)	Gill	0.396 ± 0.184 (5)	—
	Mantle	0.006 ± 0.002 (5)	—
Laboratory maintained (1 mo)	Gill	0.092 ± 0.021 (5)	—
	Mantle	not detected	—

* Data from Lee and Childress (1994), 50 μ*M* ^{15}N substrate.

tion of collection and maintenance. In 1993 we attempted to minimize potential effects of maintenance history. Mussels were incubated very soon after collection in shipboard experiments, and mussels for Santa Barbara experiments were collected during one of the last dives of the cruise to reduce the period of maintenance aboard ship. Laboratory experiments in 1993 were conducted primarily on mussels collected at Brine Pool NR-1. These mussels had a conspicuously higher degree of gill hypertrophy (Lee, pers. obs.) that may have reflected a greater number of symbionts, better host condition, or both.

Effects of external ammonium and nitrate concentration on N assimilation

The effects of substrate concentration on N assimilation are shown in Fig. 3a–c. Gills from individual mussels were subdivided into several pieces and incubated in the presence of increasing concentrations of $^{15}NH_3$ or $^{15}NO_3$-. Rates of ^{15}N assimilation at 50 μ*M* substrate concentrations were typical of rates observed for $^{15}NH_3$ and were among the highest observed for $^{15}NO_3$- by excised gills. The rate of ^{15}N assimilation increased with substrate concentration in both treatments, with apparent saturation at concentrations of 200 μ*M*. Rates were comparable between $^{15}NH_3$ and $^{15}NO_3$- treatments at 50 μ*M* substrate concentrations. At high substrate concentrations, rates of $^{15}NH_3$ assimilation were greater than rates observed for $^{15}NO_3$- assimilation. The effect of increased $^{15}NH_3$ concentration on ^{15}N assimilation by whole mussels was also investigated by using intact mussels collected the same year. At high $^{15}NH_3$ concentrations, rates of assimilation into gill tissue of whole mussels were similar to rates obtained from excised gills; however, rates at 10 and 50 μ*M* $^{15}NH_3$ were higher in whole mussels. These results indicate that affinity for ammonium is higher in whole mussels than in excised gills. The apparent K_s values for ammonium were 24 μ*M* for excised gills and 10 μ*M* for gills from intact mussels.

Internal $^{15}NH_3$ in $^{15}NH_3$ incubations

In a time-course experiment using excised gill pieces incubated with 50 μ*M* $^{15}NH_3$, assimilated ^{15}N and internal $^{15}NH_3$ increased as a function of time (Fig. 4a, b). A maximum value of about 0.6 m*M* $^{15}NH_3$ was attained in 2 h.

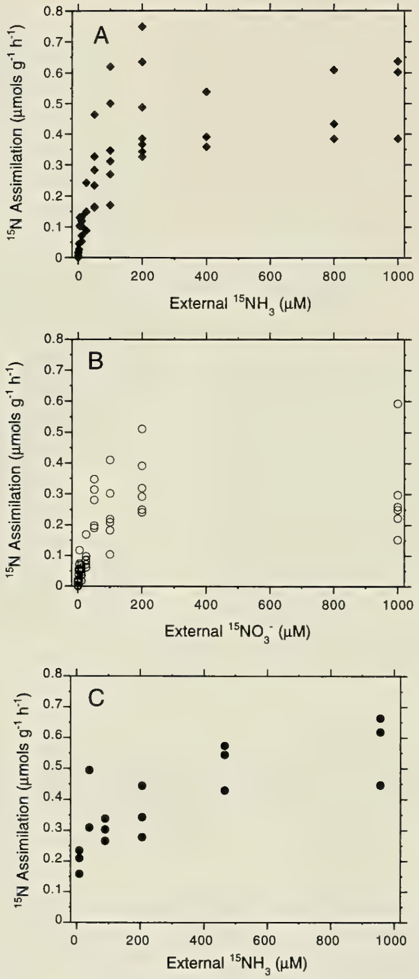

Figure 3. ^{15}N assimilation as a function of ^{15}N substrate concentration in the medium. (A) $^{15}NH_3$ incubation, excised gill (1992 collection, 6 individuals, 3- or 6-h incubation). (B) $^{15}NO_3$- incubation, excised gill (1992 collection, 6 individuals, 3- or 6-h incubation). In A–B, each data point represents a single gill-piece incubation. (C) $^{15}NH_3$ incubation, gill from whole mussel (1992 collection, 17 individuals, 6-h incubation).

The appearance of internal $^{15}NH_3$ was consistently observed in $^{15}NH_3$ incubations. $^{15}NH_3$ assimilation rate correlated with internal $^{15}NH_3$ concentration in gills of whole mussels exposed to 50–100 μM $^{15}NH_3$, excised gills exposed to 0–1000 μM $^{15}NH_3$, and gills from whole mussels exposed to 10–1000 μM $^{15}NH_3$ (Fig. 5a–c). The relation between internal $^{15}NH_3$ and ^{15}N assimilation rate differed between these experiments. No saturation of ^{15}N assimilation as a function of internal $^{15}NH_3$ was observed. In comparison with the medium, internal concentrations of $^{15}NH_3$ were considerably elevated in gills from whole mussels and generally elevated in excised gills (Fig. 6a, b). Gills from whole mussels showed a clear relation between internal and external $^{15}NH_3$. In mantle tissue, internal $^{15}NH_3$ increased as a function of external $^{15}NH_3$ but was not elevated compared with the medium. These findings indicate that a mechanism for concentrating ammonium into the gill operates in the intact association.

Internal $^{15}NH_3$ in $^{15}NO_3$- incubations

Excised gill pieces incubated with 50 μM $^{15}NO_3$- exhibited increased incorporation of ^{15}N as a function of time (Fig. 7a). Rates of ^{15}N incorporation were lower than observed in a parallel time-course experiment involving $^{15}NH_3$ assimilation by gill pieces from the same mussel

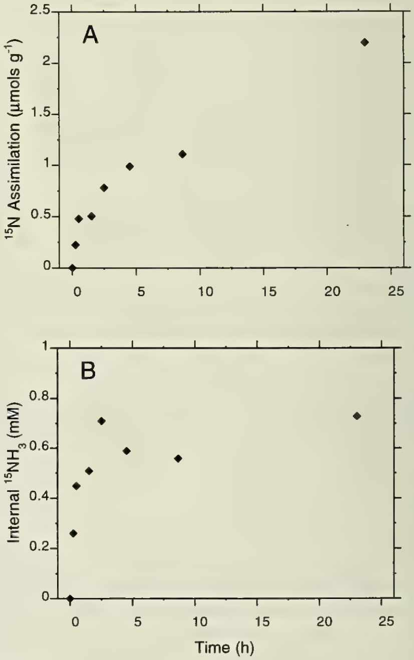

Figure 4. Time-course of $^{15}NH_3$ incorporation as (A) organic material and (B) unassimilated $^{15}NH_3$ by pieces of excised gill (1992 collection, 1 individual).

(Fig. 4a). $^{15}NH_3$ was detected in gills incubated in $^{15}NO_3$- and was found to increase with time to a maximum value of around 50 μM (Fig. 7b). Concentrations of internal $^{15}NH_3$ were moderate in all incubations in which $^{15}NO_3$- assimilation was exhibited. When excised gill pieces were incubated with $^{15}NO_3$- in the range of 0–2000 μM for 3 or 6 h, internal $^{15}NH_3$ reached concentrations up to several hundred micromoles (Fig. 8) and appeared to attain maximum values in the presence of 100–200 μM $^{15}NO_3$-. The internal $^{15}NH_3$ of gills that exhibited negligible $^{15}NO_3$- assimilation also increased as a function of external $^{15}NO_3$-, but the concentrations of $^{15}NH_3$ were substantially lower. As with $^{15}NH_3$-incubated gills, ^{15}N assimilation by $^{15}NO_3$--incubated gills was correlated with internal $^{15}NH_3$ concentration (Fig. 9a). This was the case for excised gills that had moderate rates of assimilation as well as for those that had negligible assimilation. A correlation between internal $^{15}NH_3$ and assimilation of $^{15}NO_3$- was also exhibited by gills of intact mussels incubated with 100 μM $^{15}NO_3$- for 12 h (Fig. 9b). The correlations between internal $^{15}NH_3$ and $^{15}NO_3$- assimilation were similar between excised gill and whole-mussel experiments and were similar to the correlation observed for $^{15}NH_3$-incubated excised gills (Fig. 5b).

We also tested how the addition of ammonium to the medium affected $^{15}NO_3$- assimilation. Excised gill pieces incubated with $^{15}NO_3$- and increasing concentrations of $^{14}NH_3$ exhibited a decline in $^{15}NO_3$- assimilation as a function of $^{14}NH_3$ concentration (Fig. 10a). Rates of $^{15}NO_3$- assimilation were negligible in the presence of 50 μM or greater $^{14}NH_3$. Since excised gills exposed to 50 μM $^{15}NH_3$ exhibited internal $^{15}NH_3$ concentrations of 200–300 μM $^{15}NH_3$ (Fig. 6a), these results indicate that nitrate assimilation is not highly sensitive to ammonium. The concentration of internal $^{15}NH_3$ generally declined at higher $^{14}NH_3$ concentrations in the medium (Fig. 10b). The rate of $^{15}NO_3$- assimilation as a function of internal $^{15}NH_3$ was in the range exhibited for excised gills that were not exposed to $^{14}NH_3$ (Fig. 10c).

Assimilation enzymes

Nitrate reductase (NR) activity was measured in gill tissue of freshly collected mussels from the Brine Pool during dives 3539 and 3541, Bush Hill, and GC 272 (Table II). NR activity was not detected in gill tissue of Brine Pool mussels from dives 3536, 3537, and 3538 or in functionally "aposymbiotic" mussels that had been maintained in the laboratory for several months. Such mussels exhibit no detectable symbionts (Kochevar *et al.*, 1992). NR is unlikely to be of host origin, which was confirmed by the finding of negligible NR activity in mantle tissue of freshly collected mussels (Table II).

Transferase activity of glutamine synthetase (GS) was detected in gill tissue of mussels from all collections, including Brine Pool mussels that exhibited negligible NR activity (Table II). Significantly higher GS activity (ANOVA; $P < 0.05$) was measured in gills of GC 272 mussels. Mantle GS activities were generally lower than those in the gill and were significantly lower in mussels from Brine

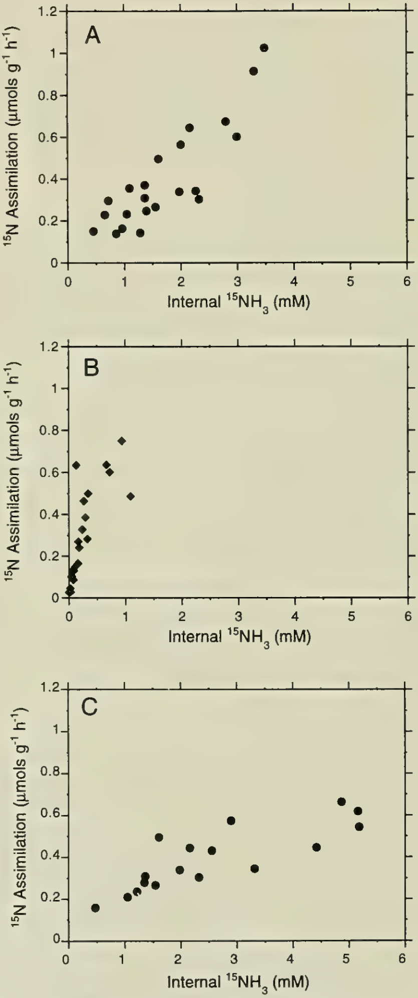

Figure 5. $^{15}NH_3$ assimilation as a function of internal $^{15}NH_3$. (A) Gills from 22 whole mussels (1991, 12- or 24-h incubation; 1993 collections, 12-h incubation) incubated in 50–100 μM $^{15}NH_3$. (B) Excised gills (1992 collection, same experiment as Fig. 3A) incubated in 0–1000 μM $^{15}NH_3$. (C) Gills from whole mussels (1992 collection, same experiment as Fig. 3C) incubated in 10–1000 μM $^{15}NH_3$.

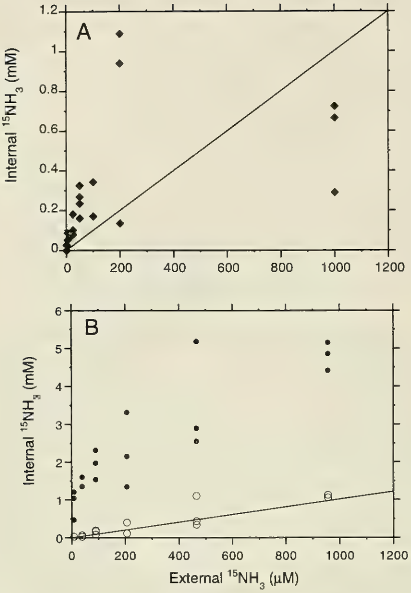

Figure 6. Internal $^{15}NH_3$ concentration as a function of external $^{15}NH_3$. (A) Excised gills (1992 collection, same experiment as Fig. 3A). (B) Whole mussels (1992 collection, same experiment as Fig. 3C). Filled circles = gill, open circles = mantle. Solid lines = unity.

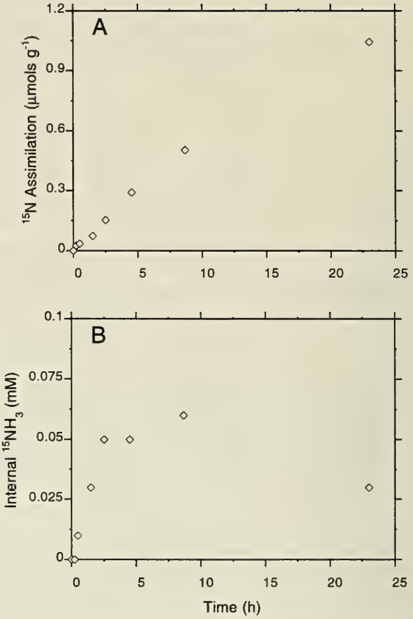

Figure 7. Time-course of $^{15}NO_3$- incorporation as (A) NaOH-treated fraction and (B) unassimilated $^{15}NH_3$ by pieces of excised gill (1992 collection, same individual as Fig. 4).

Pool Dives 3539 and 3541 and GC 272 (ANOVA; $P < 0.05$). GS activity in aposymbiotic mussels was lower than observed in freshly collected mussels and not significantly different between gill and mantle (ANOVA; $P > 0.05$).

NAD(P)H-dependent glutamate dehydrogenase (GDH) activity was detected in laboratory-maintained aposymbiotic mussels. NADH-dependent GDH activity in gill tissue was 10 times higher than NADPH-dependent activity. Activity was negligible in the absence of added ADP. NADH-dependent activity in the presence of ADP was higher in gill tissue (0.115 ± 0.069 μmol mg protein^{-1} h^{-1} (SD, $n = 7$)) than in mantle tissue (0.018, 0.027 μmol mg protein^{-1} h^{-1}). NADH- and NADPH-dependent GDH activity (with ADP present) was not detected in freshly collected seep mytilids or in symbiotic mussels maintained in the laboratory.

Discussion

Symbiotic associations can be dominant organisms in nutrient-poor tropical waters as well as in nutrient-rich sites around deep-sea vents and cold seeps. This success results in part from the ability to utilize inorganic nitrogen from the environment and recycle ammonium produced by host metabolism. However, the mechanisms involved in inorganic N uptake and assimilation by symbiotic as-

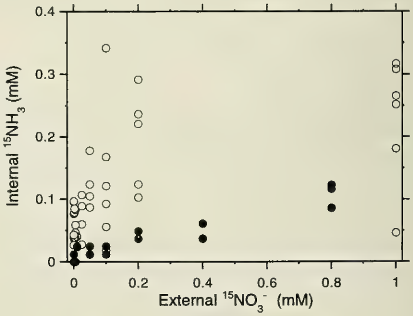

Figure 8. Internal $^{15}NH_3$ concentration as a function of external $^{15}NO_3$-. Same experiment as Fig. 2. Open circles = excised gills (1992 collection); filled circles = excised gills (1992 collection), negligible assimilation.

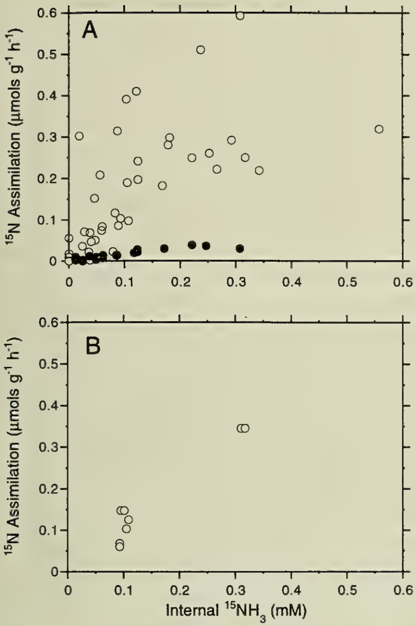

Figure 9. $^{15}\mathrm{NO}_3$- assimilation as a function of internal $^{15}\mathrm{NH}_3$. (A) Excised gills incubated with 0–1000 μM $^{15}\mathrm{NO}_3$-. Same experiment as Fig. 2. Open circles = excised gills (1992 collection), filled circles = excised gills (1992 collection), negligible assimilation. (B) Gills from 8 whole mussels (1993 collection; 12-h incubation) incubated in 100 μM $^{15}\mathrm{NO}_3$-.

sociations are not well understood. In studies involving algal–invertebrate symbiosis, the endosymbiont has generally been considered to be the partner that facilitates uptake and assimilation of inorganic nitrogen. This is most straightforward because marine invertebrates are believed not to take up or assimilate ammonium to a great extent, and they lack nitrate reductase.

Because the endosymbionts are not in direct contact with the medium, they cannot take up nutrients from the environment directly. As a result, the "barrier" of the host organism must be taken into account when considering mechanisms of nutrient uptake by symbiotic associations. One model of uptake and assimilation proposed for algal-invertebrate associations is the "depletion–diffusion" mechanism (D'Elia and Cook, 1988). Such a mechanism requires that low concentrations of ammonium and nitrate be maintained within the host tissue by active uptake of nutrients by the symbionts. Concentrations of inorganic N in the host tissue of algal–invertebrate associations are believed to be in the micromolar range (Falkowski, *et al.*, 1993) and sufficiently low to facilitate diffusive influx from the environment. Although few measurements have been made, this mechanism is supported by direct measurements of internal ammonium levels such as those by Crossland and Barnes (1977) and Wilkerson and Muscatine (1984).

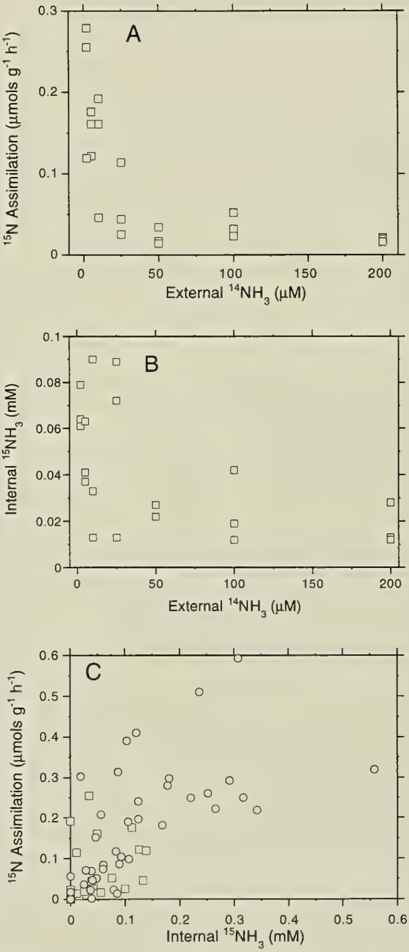

Figure 10. Effect of 2–200 μM $^{14}\mathrm{NH}_3$ on $^{15}\mathrm{NO}_3$- incorporation by excised gills (1992 collection, 3 individuals). (A) $^{15}\mathrm{NO}_3$- assimilation *vs.* $^{14}\mathrm{NH}_3$ concentration. (B) Internal $^{15}\mathrm{NH}_3$ concentration *vs.* $^{14}\mathrm{NH}_3$ concentration. (C) $^{15}\mathrm{NO}_3$- assimilation as a function of internal $^{15}\mathrm{NH}_3$—comparison between $^{14}\mathrm{NH}_3$/$^{15}\mathrm{NO}_3$- (squares) and $^{15}\mathrm{NO}_3$- incubations (open circles). Each data point represents a single gill-piece incubation.

Table II

Activities of nitrate reductase and glutamine synthetase in seep mussels; unless otherwise given, values are ± SD (n)

	Activity (μmol substrate mg protein^{-1} hour^{1})	
	Nitrate reductase	Glutamine synthetase
Brine Pool Dives 3536, 3537, 3538		
Gill	0.047 ± 0.038 (9)	2.909 ± 0.985 (9)
Mantle	—	0.563 ± 0.284 (9)
Brine Pool Dives 3539, 3541		
Gill	0.132 ± 0.140 (12)	2.588 ± 1.097 (8)
Bush Hill		
Gill	0.141 ± 0.105 (12)	2.569 ± 1.187 (10)
Mantle	—	1.627, 2.926
GC 272		
Gill	0.143 ± 0.061 (7)	4.270 ± 1.029 (4)
Mantle	0.032 ± 0.023 (3)	0.704 ± 0.503 (4)
Aposymbiotic		
Gill	−0.003 ± 0.028 (10)	0.721 ± 0.675 (4)
Mantle	0.001 ± 0.017 (5)	0.347 ± 0.445 (5)

In the seep mytilid, in contrast to algal–invertebrate associations, millimolar concentrations of ammonium were measured using a method that is quantitative and specific for $^{15}NH_3$ (Fig. 6a, b). The presence of such levels is not consistent with a depletion–diffusion mechanism unless the concentrations observed (up to 5 mM $^{15}NH_3$; Fig. 6b) are due primarily to sequestration of ammonium by the symbionts, which compose roughly 11%–14% of gill filament cell volume. These findings indicate that host-mediated mechanisms of ammonium transport may be present. This theory is supported by the observation that ammonium uptake by seep mytilids appears to conform to Michaelis–Menten kinetics rather than to the linear kinetics expected for a diffusive mechanism (Lee *et al.*, 1992b). Such a capacity would reflect adaptation by the host to the chemosynthetic lifestyle of the symbiont. Elevated concentrations of internal ammonium appear to be common in associations between marine invertebrates and chemosynthetic bacteria (Lee *et al.*, 1992a; Lee, unpub. data). Thus bacterial symbionts are apparently subject to elevated nitrogen availability. This has important implications for nutritional interactions and assimilation pathways.

Internal ammonium and ammonium assimilation

Unlike methane, which is probably not concentrated in mussel tissues, ammonium can be sequestered. Thus, ammonium assimilation is not directly dependent on immediate availability in the environment. Gills of mussels exposed to 100 μM $^{15}NH_3$ exhibited about 2 mM internal $^{15}NH_3$ (Fig. 6b) and 0.3 μmol g^{-1} h^{-1} $^{15}NH_3$ assimilation (Fig. 3c). At that rate, 7 h would be required for all the stored ammonium to be assimilated. Because concentrations of ammonium within seep mytilid gill tissues were high and $^{15}NH_3$ taken up from the medium appeared in the internal ammonium pool (Figs. 4a, b and 6a, b), it is likely that concentrations of internal ammonium rather than external ammonium were more directly relevant to ammonium assimilation. This conclusion is supported by the finding that the rate of $^{15}NH_3$ assimilation was correlated with internal $^{15}NH_3$ concentration (Fig. 5a–c).

Two possible pathways are likely to facilitate assimilation of ammonium into amino acids: glutamate dehydrogenase (GDH) and glutamine synthetase/glutamate synthase (GS/GOGAT). One difference between the GDH and GS/GOGAT pathways is the affinity for ammonium (Miflin and Lea, 1977). GDH exhibits K_m values for ammonium in the millimolar range, whereas GS has a considerably higher affinity, favoring the functioning of GS when ammonium concentrations are low. In marine symbiosis studies, GDH is of interest because it is an enzyme that is present in host tissue and may be involved in assimilation of ammonium by the host. In algal–invertebrate symbiosis, host (cnidarian) GDH exhibits a low affinity for ammonium, with K_m values for ammonium of NADP-dependent GDH in the micromolar range (Male and Storey, 1983; Catmull *et al.*, 1987; Rahav *et al.*, 1989). GS may also be present in host tissue and may enable the host to assimilate ammonium in algal-invertebrate associations (*e.g.*, Rees, 1986; Rees *et al.*, 1994; McAuley, 1995). Given the finding of millimolar concentrations of internal ammonium in the seep mytilid, GDH might function in the assimilation of ammonium, but GDH activity was not detected in symbiotic mussels. Therefore, GS/GOGAT is the most plausible assimilation pathway.

Relatively low activities of ammonium-assimilation enzymes were observed in the seep mytilid. In the green hydra symbiosis, in which the host is believed to assimilate ammonium by GS, GS activities are an order of magnitude higher than observed in seep mytilid gill (Rees, 1986). GS activities of the giant clam (*Tridacna gigas*) symbiosis were up to 30 times higher (Rees *et al.*, 1994). The GDH activities reported here for the aposymbiotic seep mytilid are in the range of GDH activities for a nonsymbiotic mussel, *Geukensia demissa* (Reiss *et al.*, 1977), and for host tissue of the coral *Stylophora pistillata* (Rahav *et al.*, 1989), but they are one to two orders of magnitude lower than those observed in host tissue of the green hydra and the coral *Acropora formosa* (Rees, 1986; Catmull *et al.*, 1987). The finding of lower GS activity in particular indicates that assimilation enzymes in the seep-mytilid symbiosis are at lower concentration than in algal–invertebrate associations and may reflect higher internal concentrations of substrate.

Internal ammonium and nitrate assimilation

Ammonium is assimilated preferentially over nitrate by bacteria and algae (Syrett, 1981; Merrick, 1988) and inhibits assimilatory nitrate reduction (Guerrero *et al.*, 1981; Solomonson and Barber, 1990). It is not clear how nitrate is assimilated by seep mytilids when concentrations of ammonium within the tissues are high. One possibility is that dissimilatory pathways are used to reduce nitrate to ammonium. Dissimilatory (or respiratory) nitrate reduction, in which nitrate acts as an alternative electron acceptor to oxygen, is not regulated by ammonium and occurs at rates in excess of autotrophic N demands (Cole, 1988). Dissimilatory nitrate reduction has been documented in several thiotrophic bacteria–invertebrate symbioses (Wilmot and Vetter, 1992; Hentschel *et al.*, 1993; Hentschel and Felbeck, 1993). However, results from these studies are not consistent with the functioning of classical dissimilatory pathways (Nelson and Hagen, 1995). Nitrate reduction in seep mytilids appeared to exhibit characteristics of both assimilatory and dissimilatory nitrate reduction. Consistent with dissimilatory nitrate reduction is our finding that seep mytilids utilized nitrate despite a large internal ammonium pool. This pool was observed in the present study as well as in freshly collected mussels and mussels kept without added ammonium (Lee and Childress, in prep.). Nitrate reduction resulted in the production of ammonium in excess of assimilation since $^{15}NH_3$ was produced during $^{15}NO_3$- assimilation (Fig. 7b, 8). Although dissimilatory nitrate reduction has not been demonstrated in free-living methanotrophs, further evidence for a dissimilatory pathway comes from the finding that cytochrome *b* of the seep mytilid symbiont was oxidized by nitrate (D. W. Kraus *et al.*, Univ. Alabama at Birmingham, in prep). Inconsistent with a dissimilatory pathway is our finding that addition of $^{14}NH_3$ to the medium resulted in a slight inhibition of nitrate reduction (Fig. 10b).

Most of the possible end products of dissimilatory nitrate reduction (nitrite, NO, N_2O, or N_2) are not readily incorporated into amino acids. Therefore, nitrate utilized in symbiont respiration cannot be used as a source of nitrogen unless the end product is ammonium—*i.e.*, unless there is dissimilatory nitrate reduction of ammonium (DNRA). In our experiments, $^{15}NH_3$ appeared internally following exposure to $^{15}NO_3$-. Assimilation of ^{15}N from $^{15}NO_3$- was correlated with internal $^{15}NH_3$ concentration. We propose that the major end product of the putative dissimilatory pathway in seep mytilid la is ammonium, which is retained in the association and subsequently assimilated.

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Comparison of Temperature Effects on Heart Performance of the Dungeness Crab, *Cancer magister*, *in vitro* and *in vivo*

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Abstract. A large percentage of physiological studies are based on isolated components of complex systems, but the question can always be posed, are the responses the same in isolation as when these components are under the homeostatic controls that exist *in vivo?* For cardiac performance in *Cancer magister,* the responses to temperature variation over the range 4° to 20°C are different in semi-isolated hearts than in intact animals. Cardiac performance in semi-isolated hearts was measured with a pressure transducer, a flow transducer, and electromyogram (EMG) electrodes, and in intact animals with pulsed Doppler flow probes. Heart rate increase in semi-isolated hearts was about one-third of that in intact animals. The cardiac output of semi-isolated hearts decreased with increasing temperature, whereas that of intact animals increased. Stroke volume decreased linearly in semi-isolated hearts. In intact animals, stroke volume decreased from 4° to 12°C, but remained relatively stable from 12° to 20°C. The ventricular pressure and the EMG amplitude of semi-isolated hearts both decreased with increasing temperature. Double systolic contractions appeared both in semi-isolated hearts and in intact animals in the temperature range 13° to 20°C; this may represent a compensatory mechanism at extreme temperatures. The difference in cardiac performance between intact crabs and semi-isolated hearts reflects, almost certainly, extrinsic control in intact animals, including modulation by cardioregulatory nerves or neurohormonal modulation of the cardiac ganglion, myocardial contractility or changes in outflow resistance.

Introduction

To analyze the complex workings of the crustacean open circulatory system, investigators have frequently turned to the study of isolated components of the system. We will show here that this approach, while revealing the fundamental responses of these isolated parts, may fail to provide insight into the performance of the whole system with all of its integrative mechanisms intact.

Cardiac performance in crustaceans can be described by a set of characteristics, and the most important physiologically is cardiac output, the product of heart rate and stroke volume. Heart rate is controlled intrinsically by the cardiac ganglion situated in the inner dorsal wall of the heart (Alexandrowicz, 1932; Maynard, 1960). Heart rate is also controlled extrinsically by the cardioaccelerator (CA) and cardioinhibitor (CI) nerves, which connect the central nervous system to the cardiac ganglion and possibly to the myocardium (Florey, 1960; Field and Larimer, 1975; Wilkens and Walker, 1992), and by neurohormones released from the pericardial organs (Cooke and Sullivan, 1982; Cooke, 1988; Wilkens, 1987; Wilkens and McMahon, 1992). Stroke volume is regulated both by diastolic filling and by systolic ejection, the latter influenced by myocardial contractility and by peripheral resistance. The regulation of crustacean heart performance has been the subject of several articles (see McMahon and Burnett, 1990; Kuramoto and Yamagashi, 1990; Wilkens and McMahon, 1992), but understanding of the system is far from complete, particularly with reference to the effects of ambient temperature on the various neuronal and hormonal control mechanisms.

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Abbreviations: f_H, heart rate; $\dot{Q}$, cardiac output; V_s, stroke volume; P_{vent}, ventricular pressure.

The effect of temperature on the crustacean circulatory system has mostly been determined by measuring heart rate, the easiest of all circulatory parameters to measure, in intact animals (see review by Maynard, 1960, and McMahon and Wilkens, 1983). In a variety of crustaceans, heart rate increases with temperature to yield Q_{10} values between 4 at low temperatures and 1.5 at high temperatures (Ahsanullah and Newell, 1971; Florey and Kriebel, 1974; Spaargaren, 1974; Spaargaren and Achituv, 1977; McMahon *et al.,* 1978; deFur and Mangum, 1979; Burton *et al.,* 1980; Depledge, 1984; Zainal *et al.,* 1992; review by McMahon and Burnett, 1990). But these observations on heart rate are of limited value, because the other component of cardiac output, stroke volume, has rarely been measured even though it can be varied independently (Jorgensen *et al.,* 1982; Bourne *et al.,* 1988; Airriess and McMahon, 1994; McGaw *et al.,* 1994a, b).

In this study we measured the direct effects of temperature on the myocardial performance and cardiac ganglion of semi-isolated hearts of *Cancer magister.* The potential roles played by extrinsic cardiac control systems in responses to temperature were evaluated by comparison with the cardiac responses of intact animals.

Materials and Methods

In vivo *measurements*

In vivo measurements were carried out at the Bamfield Marine Station, British Columbia, Canada. Adult male specimens of *Cancer magister* (weight range 500–765 g) were purchased from local suppliers and held in tanks with aerated, running, natural seawater. Water temperature in the holding tanks over the experimental period (September to January) was 12° ± 1°C, and salinity was 32 ± 2 ppt. The crabs were held for up to 4 weeks prior to experimentation. During this period, animals were fed fish twice a week. They were starved for 2 days before and during experimental recordings.

The chamber used for recording consisted of an insulated polystyrene container that was small enough to restrict the crab's movement. An overhead tank was used to maintain a constant seawater flow and thoroughly aerate all incoming seawater. Bath seawater temperature was adjusted by passing it through a glass coil in a water bath (Haake T41). A valve system allowed either control or test water to be delivered to the chamber. Animals were acclimated at 12°C and then subjected to either a temperature increase to 20°C or a decrease to 4°C. Temperature transitions were completed in less than 45 min, with a temperature change of $0.1°–0.3°C \cdot min^{-1}$.

Cardiac output ($\dot{Q}$), stroke volume (V_s) and heart rate (f_H) were assessed with a pulsed Doppler flow meter (University of Iowa, Bioengineering) by the method developed by Airriess *et al.* (1994) and by Airriess and McMahon (1994). Airriess *et al.* (1994) verified these techniques for accurate measurement of blood flow in equivalent-sized *C. magister* and showed a high correlation between actual flow and perfusion rate ($R^2 = 0.996$). When the pulsed Doppler flow-meter technique is used, handling stress to the animals is reduced, and values for cardiac output and stroke volume are therefore considerably lower than estimations based on the Fick principle (Airriess and McMahon, 1994; Airriess *et al.,* 1994; McGaw *et al.,* 1994a). Flow through the anterior median and anterior lateral arteries was measured noninvasively; flow through the dorsal abdominal and sternal arteries was measured by inserting the Doppler probes into the body cavity through polyethylene catheters (o.d. 0.9 mm, i.d. 0.6 mm). Hemolymph loss was prevented by inserting the catheter through latex dental dam that was glued over the hole site. The probes were held in place with commercial cyanoacrylate glue and dental periphery wax. In these experiments, no recordings were made from the hepatic arteries because of their location deep in the thorax and the trauma caused by probe placement. The hepatic artery is known to deliver only a small part of the cardiac output, and hemolymph flow showed no major changes during exposure to hypoxia or neurohormone injections (Airriess and McMahon, 1994; McGaw *et al.,* 1994b). Measurement of center stream velocity was accomplished by lateral positioning of the probe in combination with vertical focusing of the Doppler signal.

Flow-meter output and seawater temperature were recorded continuously by a computerized data acquisition system. Raw data were sampled at 100 Hz per channel by a data acquisition board (National Instruments ATMIO16X), displayed in real time, and simultaneously stored to hard disk. The computer program was developed in-house using the Labview data acquisition package (National Instruments). Data transformations consisted of median filtering (rank = 10) to improve the signal-to-noise ratio and calculation of the actual flow or seawater temperature. Flow-meter output (0.5 V = 1 kHz Doppler shift) was converted to hemolymph flow with the modified Doppler equation. Arterial radii of crabs showed no statistically significant correlation with weight of the animals over the limited size range used, and the following mean arterial radius values were therefore used (McGaw, unpub. data): anterior median = 0.58 mm; anterioral lateral = 0.74 mm; sternal = 1.56 mm; dorsal abdominal = 0.61 mm. Heart rate was determined by counting the flow peaks on one of the flow channels. Approximate cardiac output was calculated as the sum of all arterial flows. Flow through each hepatic artery was assumed equal to $8\ \mu l \cdot s^{-1}$ ($0.5\ ml \cdot min^{-1}$), an average value reported for this artery in similarly treated *C. magister* at 12°C (Airriess and McMahon, 1994).

After placement of the probes, which involved about 60 min of handling in a shallow seawater-filled pan, animals were allowed to recover in the experimental chamber at their acclimation temperature for at least 18 h. This period was sufficient for the animals to recover from handling and surgery and provided baseline measurements (Airriess and McMahon, 1994). Experimental recording periods lasted about 6 h. Baseline measurements were recorded minimally for 30 min at the initial acclimation temperature before temperature change commenced. Temperature was held constant after the test temperature was reached.

In vitro *measurements*

Adult male *C. magister* were purchased from a local fish supplier in Calgary, Alberta. They were held in large aquaria with aerated, recirculating seawater (Instant Ocean, 32 ± 2 ppt, 12° ± 1°C) for at least 2 weeks to ensure their health prior to experimentation. Animals were fed previously frozen smelt twice a week. Preparations of semi-isolated heart were made following the method of Wilkens and Mercier (1993). The experimental protocol was as follows: The heart was rapidly dissected out, but it was left attached to the dorsal side of the carapace, and the alary ligaments and pericardial septum were kept intact. Cardioregulator nerves were cut and the preparation was washed with saline. The hearts were submerged in *Cancer* saline: NaCl 468 m*M;* $CaCl_2 \cdot 2H_2O$ 11 m*M;* KCl 9 m*M;* $MgSO_4 \cdot 7H_2O$ 13 m*M;* $MgCl_2 \cdot 6H_2O$ 9 m*M;* $NaHCO_3$ 2 m*M;* glucose 5 m*M;* Hepes 10 m*M;* pH 7.6; 10°C (modified after Morris and McMahon, 1989). Saline was aerated throughout the experiment. Temperature of the water bath was controlled by a water jacket connected to a thermostated water bath (Lauda RM6). Temperature of the circulating saline was measured with a YSI 400 thermocouple probe and a YSI tele-thermometer.

After placing the heart ventral side up in the experimental dish, the different probes were installed. Heart rate was measured with a rate analyzer (Gould ECG amplifier). Ventricular pressure (P_{vent}) was measured with a pressure transducer (HP 276BC) connected to a cannula inserted through one of the hepatic arteries into the ventricle. Pressure signals were amplified by an HP 311A transducer amplifier. Electromyogram (EMG) recordings were obtained with a wire electrode inserted into the dorsal heart wall. This wire was secured by covering it with a small patch of dental rubber sheet glued to the connective tissue layer with commercial cyanoacrylate glue. No damage due to the use of this glue was apparent. The indifferent electrode was placed in the pericardial sinus outside the heart. Signals were preamplified with a Grass P15 AC preamplifier. The amplitude of the first peak, with the amplitude at the start of observations at 12°C assigned a value of 1 (marked with an asterisk on Fig. 11), was measured as an indication of the magnitude of the initial burst of spikes from the cardiac ganglion. Anterior median, anterior lateral, dorsal abdominal, and one hepatic artery were tied off to force all flow through the sternal artery. When all outflow was restricted to the sternal artery, P_{vent} was 1.2 kPa. For related intact crabs of similar size, such as *Cancer productus,* P_{vent} is in the range of 1–2 kPa (McMahon and Wilkens, 1983).

Cardiac output was measured with a flow transducer (Biotronex Laboratory 610 pulsed logic flow meter) connected to the cannula inserted in the sternal artery. The cannula was led back into the water bath at zero afterload. Previously published data confirm that this heart preparation is not damaged by ligature of the arteries and shows only passive responses of the heart performance to changes in preload, afterload, and outflow resistance (Wilkens, 1993; Wilkens and McMahon, 1995). The flow transducer was tested frequently by weighing the volume of saline pumped over a 30-s period. All electrical signals were fed into an oscilloscope (Tektronix 5130N) and simultaneously recorded on paper (Gould Brush 2400) and on tape (HP 3964A instrumentation tape recorder). Stroke volume was calculated by dividing cardiac output by heart rate. The pressure waveform of the semi-isolated heart was analyzed on a Nicolet 4094. Acute Q_{10} was calculated as $Q_{10} = (f_{H2}/f_{H1}) \exp(10/t_2 - t_1))$ where f_{H2} is the heart rate at temperature t_2 and f_{H1} is the rate at temperature t_1.

Temperature was changed in a cyclic manner at 0.2°–0.4°C · min^{-1} through at least two complete temperature cycles. One cycle was defined as temperature from the starting point to the lowest and highest point and then back to the starting temperature. Heart rates of these preparations remained steady for 4 to 5 h. Hearts were continuously perfused (1 ml · min^{-1}) through a cannula inserted directly into the ventricle. This represented about 10% of the cardiac output at 12°C, but helped maintain heart viability by providing a continuous supply of oxygen and glucose, particularly at high temperatures when cardiac output dropped to zero.

The percent cumulative filling during diastole was determined by reversing the sign of the coefficient of the nonlinear regression equation of the form $(a - b \cdot x) \exp(-c \cdot x)$ fitted to the pressure wave (Sigmaplot, Jandel). This assumes that the inverse of the negative pressure during diastole is proportional to filling.

Data were collected from 10 intact animals and 7 semi-isolated hearts. Statistical analyses were taken from all 10 intact animals and the last 5 semi-isolated hearts. Statistical analysis consisted of correlation and regression analysis.

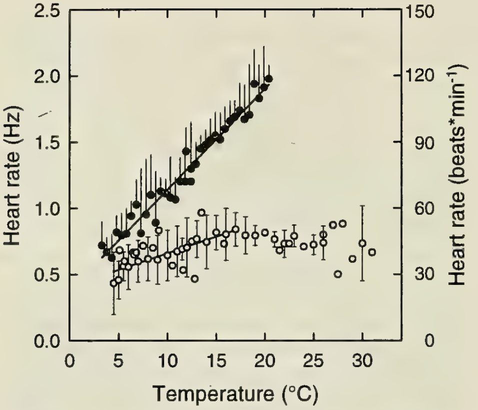

Figure 1. Heart rate of semi-isolated hearts (open circles) and intact *Cancer magister* (closed circles) as a function of temperature. Mean values ± SD. Superimposed are the regression lines for both series of data.

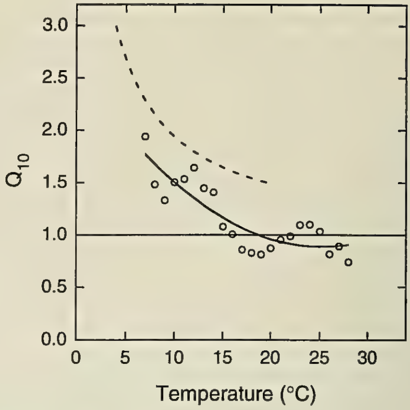

Figure 2. Q_{10} values for semi-isolated hearts (solid line and open circles) and intact *Cancer magister* (dashed line) are shown as a function of temperature. Lines are calculated by a second-order regression equation.

Results

Heart rate is intact animals showed a linear relationship with temperature up to 20°C. Heart rate of semi-isolated hearts also showed a positive but different linear relationship with temperature up to about 18°C (Fig. 1). At higher temperatures, this correlation disappeared ($P = 0.439$). Heart rate in the semi-isolated hearts was always lower than in intact animals, and the difference increased with increasing temperature. The regression equation for the heart-rate–temperature relationship was $1.51 \cdot T + 24.58$ ($P < 0.001$) for semi-isolated hearts (4°–18°C); it was $4.52 \cdot T + 22.04$ ($P < 0.001$) for intact animals (T is temperature in degrees Celsius). The rise in heart rate in semi-isolated hearts was thus about one-third that in intact animals, whereas the extrapolated heart rates at 0°C were similar. Acute Q_{10} of the semi-isolated hearts was always below 2, and it decreased with increasing temperature. The Q_{10} in the high temperature range was around 1. Heart rates of semi-isolated hearts recovered fully at 12°C after two full temperature cycles, regardless of whether temperature was first increased or decreased. In intact animals, the Q_{10} decreased from 3 at 4°C to 1.5 at 20°C (Fig. 2).

In the semi-isolated hearts, the cardiac-output–temperature relationship was negative (Fig. 3 and Table I). The cardiac output of semi-isolated hearts decreased significantly with temperature increase ($P < 0.001$) and approached 0 $\text{ml} \cdot \text{min}^{-1}$ at temperatures above 20°C. At the highest temperatures the muscles of the myocardium and of the ostial valves appeared to fail to contract and perfusate exited *via* the ostia, as confirmed by the roiling of the shallow layer of saline over them. In one semi-isolated heart, a positive relationship was noted between cardiac output and temperature. We have no explanation for this exceptional case. In contrast with these results, cardiac output in intact animals increased with increasing temperature up to 20°C (Fig. 3).

Stroke volume, which was calculated from heart rate and cardiac output, decreased similarly and significantly

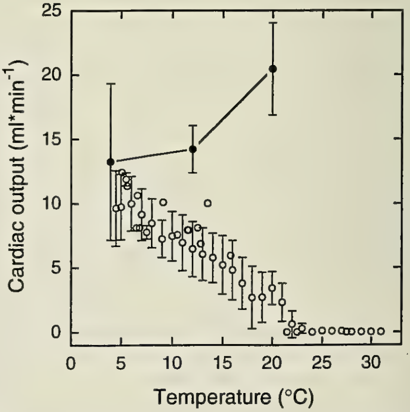

Figure 3. Cardiac output of semi-isolated hearts (open circles) and intact *Cancer magister* (solid circles) as a function of temperature. Mean ± SD.

Table I

Correlation values and their significances between the different variables measured in isolated hearts of Cancer magister

Correlation	Temp range	P_{vent}	EMG	f_H	$\dot{Q}$	V_s
T	5°–30°C	−0.7317*** (219)	−0.4542*** (199)	0.3691*** (219)	−0.8568*** (186)	−0.7823*** (186)
	5°–20°C	−0.5076*** (186)	−0.2177** (166)	0.4840*** (186)	−0.7199*** (153)	−0.7079*** (153)
P_{vent}	5°–30°C		0.5634*** (199)	−0.2727*** (199)	0.6172*** (186)	0.5521*** (186)
	5°–20°C		0.4600*** (166)	−0.2745*** (166)	0.3173*** (153)	0.3143*** (153)
EMG	5°–30°C			−0.3275*** (199)	0.5484*** (170)	0.5524*** (170)
	5°–20°C			−0.3147*** (166)	0.4638*** (137)	0.4870*** (137)
f_H	5°–30°C				−0.1444* (186)	−0.6789*** (186)
	5°–20°C				−0.0585 ns (153)	−0.7479*** (153)
$\dot{Q}$	5°–30°C					0.7515*** (186)
	5°–20°C					0.6089*** (153)

EMG, electromyogram amplitude; f_H, heart rate; P_{vent}, ventricular pressure; $\dot{Q}$, cardiac output; T, temperature; V_s, stroke volume; $P < 0.001$ = ***, $P < 0.01$ = **, $P < 0.05$ = *, $P > 0.05$ = ns. Values for cardiac output and stroke volume for the one experiment where flow increased with temperature were not incorporated in these calculations. The number of datapoints is given in brackets.

with temperature over the range 4°–12°C ($P < 0.001$) in both semi-isolated hearts and in intact crabs (Fig. 4). For the semi-isolated hearts, the regression equation was $418.81 - 18.64 \cdot T$ (T in degrees Celsius). Stroke volume of intact animals stabilized at higher temperatures.

EMG peak amplitude was measured only in semi-isolated hearts. A significant negative relationship (Table I) was found between EMG peak amplitude and temperature. EMG amplitude decreased rapidly with temperature above 20°C, until it became erratic and of very low amplitude at 30°C (Fig. 5). The effect of temperature on EMG amplitude was reversible for temperatures up to 30 °C. The decrease of EMG peak amplitude with increasing temperatures above 20°C was correlated with the loss of fluid ejection.

Ventricular pressure, which was measured only in semi-intact hearts, showed an overall negative correlation with temperature and with heart rate (Fig. 6, Table I). The pressure-temperature relationship can be divided into three phases. From 4°C to about 8°C, ventricular pressure increased, but at all higher temperatures the pressure decreased. At 20°C the pressure dropped more sharply and approached 0 as the temperature approached 30°C. Pressure was positively correlated with both EMG and cardiac output (Table I).

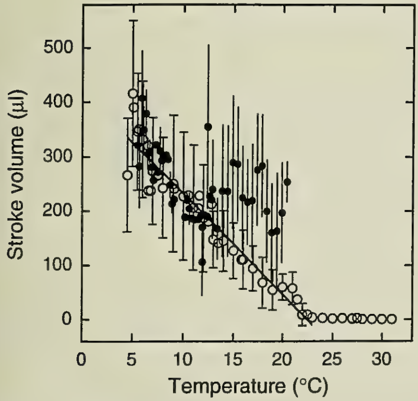

Figure 4. Stroke volume of semi-isolated hearts (open circles) and intact *Cancer magister* (solid circles) as a function of temperature. Mean ± SD.

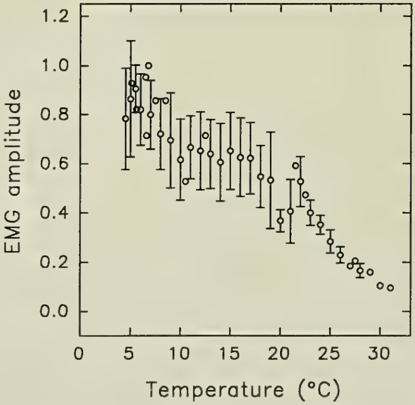

Figure 5. Electromyogram (EMG) amplitude of semi-isolated hearts of *Cancer magister* as a function of temperature. The EMG amplitude is expressed in relative units, where the amplitude at 12°C is assigned the value of 1. Mean ± SD.

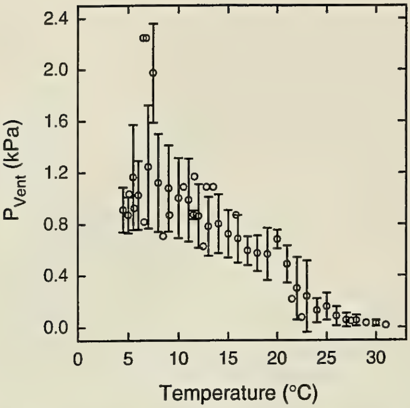

Figure 6. Ventricular pressure (P_{vent}) of semi-isolated hearts of *Cancer magister* as a function of temperature. Mean ± SD.

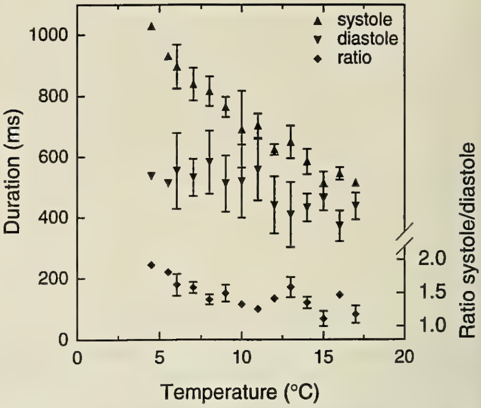

Figure 7. Duration of systole and diastole in semi-isolated hearts of *Cancer magister*, and the ratio of systolic over diastolic duration, both as a function of temperature. Mean ± SD.

When the pressure waveform was studied in greater detail, the duration of the systole was seen to decrease more (regression coefficient = −39.1 ± 2.4, $r^2 = 0.83$, $P < 0.001$) than that of diastole (regression coefficient = −12.3 ± 2.8, $r^2 = 0.63$, $P < 0.001$) as temperature increased (Fig. 7). The systolic/diastolic ratio decreased from 1.9 at 5°C to 1.1 at 17°C. Systolic positive pressure and diastolic negative pressure showed a significant positive correlation ($r^2 = 0.36$, $P < 0.05$) and consequently, the negative pressures measured during diastole showed a negative relationship with temperature (Fig. 8). Note that, although diastolic filling continues over the whole duration of diastole, 50% of the filling of the semi-isolated heart occurs during the first quarter of the diastolic period (Fig. 9). The artificial perfusion at 1 ml · min^{-1} accounts for the end diastolic pressure being slightly greater than 0.

In most experiments with semi-isolated hearts, high pressure peaks alternating with low pressure peaks suddenly began to appear at between 13° and 14°C (Figs. 10 and 11). The high and low pressure pulses seemed to alternate in a regular way. The high pressure peaks can be twice the amplitude of the low ones. The difference in amplitude decreased at temperatures above 16° to 17°C, and the high pressure peaks disappeared completely above 19° to 20°C. One semi-isolated heart, the one which showed a positive relationship between f_H and temperature, had a completely different relationship. Here double beats occurred over the whole temperature range, and the differences between low and high pressure pulses were positively correlated with temperature. In all experiments, the occurrence of high-amplitude ventricular pressure peaks coincided with the presence of a double EMG burst. When the double EMG bursts were close together, a single enlarged ventricular pressure pulse was seen. If the second EMG burst was delayed longer after the first pulse, a shoulder was observed on the pressure waveform (Fig. 11). These double systoles resulted in increased stroke volume and consequently increased cardiac output (data not shown). The negative diastolic pressure is also of greater amplitude after a double systolic beat than after a single beat. The presence of these double peaks was observed both in semi-isolated hearts and *in vivo*. No

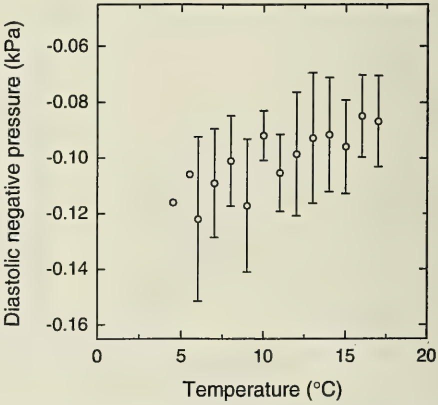

Figure 8. Maximum amplitude of ventricular negative pressure during diastole as a function of temperature for semi-isolated hearts of *Cancer magister*. Mean ± SD.

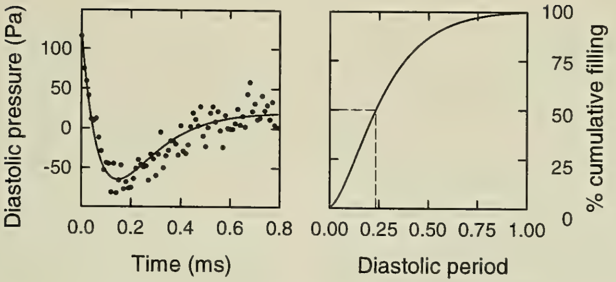

Figure 9. (A) Typical pressure waveform during diastole. Both the nonlinear regression of the form $Y = (a - b \cdot X) \cdot \exp(-c \cdot X)$ and the raw sampled data are shown. (B) Percent cumulative filling during diastole as a function of the diastolic period. The time to reach 50% filling is also shown.

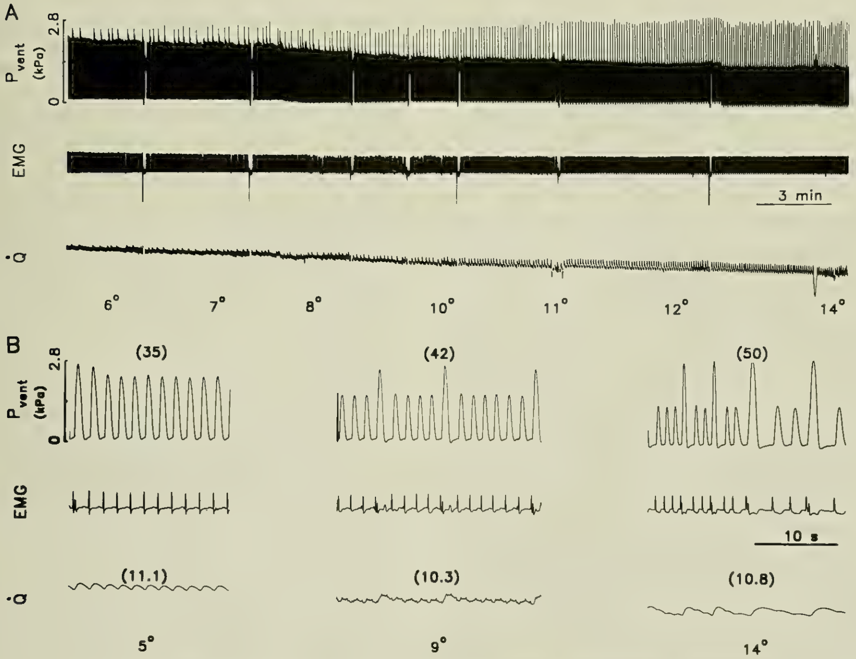

Figure 10. (A) Chart recording of ventricular pressure (P_{vent}), EMG, and cardiac output ($\dot{Q}$) from one semi-isolated heart preparation of *Cancer magister* showing the presence of double EMG bursts and increased pressure pulses at elevated temperatures. (B) Segments of the same record in A recorded at faster chart speed to show the double EMG bursts and the corresponding increases in P_{vent} and mean flow. The chart speed was doubled during the last half of the recording at 14°C. The numbers in parentheses above the pressure trace are heart rate, those above the flow trace are $\dot{Q}$ in ml • min^{-1}.

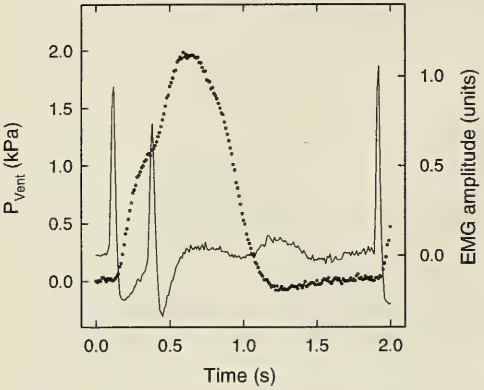

Figure 11. Digitized recording of EMG and ventricular pressure from a typical double contraction in a semi-isolated preparation of *Cancer magister.* The amplitude of the first, usually only, peak of the EMG (*) was used to construct Fig. 5.

EMG or ventricular pressure measurements were made in intact animals; but flow in the sternal artery, which makes up the majority of all flow, also showed alternating low and high peaks during temperature increases at the higher temperatures (Fig. 12). The difference between low and high amplitude flow peaks was most pronounced around 18°–19°C. The double peaks gradually disappeared during prolonged exposure to temperatures of 20°C or more.

Discussion

The components of a complex physiological system must be analyzed individually to be understood, but paradoxically the actions or responses of the parts may be different in isolation than *in vivo.* Cardiac performance in crustaceans is one such complex system, with many components each more or less independently regulated. Our assumption in this study is that the responses of semi-isolated hearts reveal the effects of temperature on the intrinsic pumping performance of the heart, whereas intact animals are able to make active adjustments to heart performance that can maintain or increase cardiac output in response to temperature stress. This assumption is supported by the observations of different cardiac responses to neurohormones in semi-isolated hearts and in intact crabs (McGaw *et al.,* 1995).

Semi-isolated hearts, although not pumping against the same afterload as in intact animals, are good models with which to observe the direct effects of perturbations such as temperature on an autorhythmic system. Our results confirm an earlier report that heart rates in semi-isolated crustacean hearts are lower than in intact animals (Wilkens and Mercier, 1993). Besides the lower heart rate, the lower Q_{10} values for semi-isolated hearts show that these preparations are less responsive to temperature change than the hearts of intact animals, suggesting that the cardiac ganglion is only partially responsible for the temperature dependence of the heart rate in intact animals. We recognize that cardiac ganglia bathed in hemolymph might respond differently to temperature than those in saline, but we had no way of testing this possibility during these studies.

The extrinsic controls that can modulate the burst rate of the cardiac ganglion may include neurohormones and the cardioregulatory nerves. In our experiments on semi-isolated hearts, the cardioregulatory nerves were not stimulated, and neurohormones were not added. Airriess and McMahon (1992) showed that injecting 10^{-7} M of octopamine and serotonin into intact crabs increased heart rate and cardiac output, whereas the responses to neurohormonal peptides were much less clear. Wilkens and Mercier (1993) showed that increased levels of the peptides crustacean cardioactive peptide (CCAP) and proctolin increased heart rate in the semi-isolated hearts of *Carcinus maenas.* In *C. magister,* however, CCAP and proctolin had little or no effect on either intact animals or semi-isolated hearts (McGaw *et al.,* 1994b, 1995). The FMRFamide-like peptides F1 and F2 produced the largest variation in effects and appear to act differently on semi-isolated heart preparations and intact animals (McGaw and McMahon, 1995; McGaw *et al.,* 1995). Stimulation of the accelerator nerves in crayfish increases heart rate (Florey, 1960; Wilkens and Walker, 1992). Thus the difference in heart rate and Q_{10} between intact animals and the semi-isolated hearts probably arises from modulation

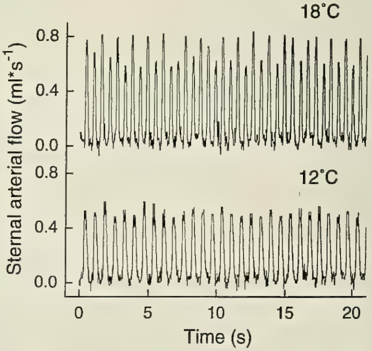

Figure 12. Patterns of hemolymph flow in the sternal artery of intact *Cancer magister* showing the presence of alternating high and low pulses at 18°C, but not at 12°C.

of the cardiac ganglion by excitatory neurons or neurohormones.

Both stroke volume and cardiac output decrease with increasing temperature in semi-isolated hearts. The decrease in stroke volume in semi-isolated hearts and in intact animals is similar in the temperature range 4°–12°C. In intact crabs, the larger increase in heart rate effectively compensates for decreased stroke volume as temperature increases, resulting in an increased cardiac output. The maintenance of stroke volume in intact animals at temperatures above 12°C seems to reflect some kind of extrinsic control. This control mechanism is, of course, absent in semi-isolated hearts. In intact *C. magister*, either serotonin or octopamine (10^{-7} *M*) increased stroke volume and cardiac output (Airriess and McMahon, 1992), and such neurohormonal modulation may be operative in intact animals. Reliable determinations of hemolymph levels of these neurohormones are still lacking, but levels in the nanomolar range appear realistic (De Wachter, unpub. data). Another untested possibility is that the afterload on the heart may be reduced in intact animals by active regulation of the arterial system. Active control of arterial resistance of the dorsal abdominal artery of crayfish has been reported (Wilkens, 1995).

The decrease in stroke volume with temperature is undoubtedly related to the decrease in ventricular pressure and systolic duration seen in semi-isolated hearts. The decrease in ventricular pressure may be due to a combination of the reduction in EMG (Fig. 5) and the direct negative effects of elevated temperature on muscle contractility. Since EMG amplitude reflects cardiac ganglion input to the myocardium, the spike firing frequency or the number of spikes per burst must have decreased with increasing temperature. This notion is supported by the finding of Jacobs and Atwood (1981) that a decrease in neural burst frequency is correlated with decreased tension in the claw opener muscle of the crayfish *Procambarus clarkii.* Similarly, increased temperature induced a reduction of muscular tension in the chelae muscles of different crustaceans (Jacobs and Atwood, 1981; Fisher and Florey, 1981; Stephens and Atwood, 1982). A conformational change in the isomerization of the actomyosin complex, which is hypothesized to generate movement, is highly temperature dependent (Taylor, 1991). Another explanation is that increases in temperature change membrane fluidity. This may influence the ion channels in the membranes and disrupt potassium and calcium levels (Prosser, 1991). In any event, our data do not allow us to speculate about the sites at which changes in temperature may exert their actions.

Analysis of the pressure waveform over the cardiac cycle showed a positive correlation between systolic and diastolic duration and between systolic positive and diastolic negative pressure. These results support the hypothesis that large-amplitude systolic contractions store more energy in the alary ligaments than do small ones, and this energy is recovered to produce larger diastolic filling.

In most of our preparations, double systolic contractions appear at about 13°C and disappear again at about 20°C. The second contraction is initiated during the pressure rise phase of the first contraction and causes an increase in both systolic pressure and stroke volume. The increase in systolic pressure is followed by an increase in the negative pressure and duration of diastole. The double contractions may result from sustained tonic firing of the small cardiac ganglion cells that feed forward to re-recruit the large cardiac ganglion cells as the latter recover from refractoriness (Cooke, 1988; Berlind, pers. com.). The cardiac ganglion appears to remain refractory for a longer period following double bursts. The appearance of double systolic pressure pulses has previously been described in isolated hearts of the lobster *Panulirus japonicus* during cooling (Kuramoto and Ebara, 1991). Kuramoto (1994) reported the appearance, upon cooling, of an increase in spike potentials and a corresponding increase in muscle contraction in isolated myocardial cells from *P. japonicus.* That this phenomenon is not the result of an artifact of the isolation procedure is supported by the observations of double contractions in intact *C. magister* hearts during acute increases in temperature above ambient (11°–12°C). These double contractions could serve to increase stroke volume without increasing heart rate. A possible mechanism *in vivo* could be the release of octopamine, since octopamine is probably released from the pericardial organ during cooling (Kuramoto and Tani, 1994). It could also be an indicator of temperature stress, since the pattern disappeared as animals acclimated to the new elevated temperature. Whether designed specifically for this purpose or not, the double cardiac ganglion bursts and resulting double systolic contractions may serve as an automatic compensatory mechanism to help overcome the negative effect of increases in temperature on stroke volume. A comparison between cardiac responses in *C. magister,* which lives in colder water (4°–20°C), and *P. japonicus* (Kuramoto, 1994), which lives in warm water (15°–25°C), suggests that the same mechanisms may occur in both of these crustaceans, but in response to opposite temperature stresses.

Above 20°C the responses of the semi-isolated hearts change. Heart rate becomes irregular, ventricular pressures and EMG amplitudes drop sharply and approach 0, and stroke volume and cardiac output fall to 0. This suggests that, although the rhythm generator still works at these temperatures, the motoneuronal bursts are less and less translated into contractions of the cardiac muscles. The disappearance of contraction at these high temperatures is not related to structural damage to enzyme systems, because a decrease of the temperature restores the heart to its original functionality. Similar heart-rate responses have been found in other intact crustaceans (see Maynard, 1960).

The data presented here support the hypothesis that control systems in these poikilothermic animals adjust their circulatory systems to the metabolic demands posed by a temperature change. Control systems that are intrinsic to the heart and others that are extrinsic are postulated. The intrinsic controls could include the direct effect of temperature on heart rate and also the increased contractions resulting from double neural bursts. The extrinsic control systems might include central nervous mechanisms operating *via* the cardioregulatory nerves, and changes in circulating levels of neuroactive substances (peptides and amines). The specific role of each of the possible extrinsic control systems is still unclear. The roles played by the different neuroactive substances and their temperature dependence, and the temperature effects on the cardioregulatory nerves remain to be elucidated.

Acknowledgments

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Biochemical and Enzymatic Characterization of an Acetylcholinesterase From *Nereis diversicolor* (Annelida, Polychaeta): Comparison With the Cholinesterases of *Eisenia fetida* (Annelida, Oligochaeta)

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Abstract. This study constitutes the first report of a biochemical characterization, involving both substrates and inhibitors and electrophoretic analysis, of a cholinesterase (ChE) from a polychaete annelid (*Nereis diversicolor*). The ChE of *N. diversicolor* appears to be an acetylcholinesterase (AChE); *i.e.,* it hydrolyzes acetylthiocholine iodide at a higher rate than other substrates and is inhibited by eserine but not by iso-OMPA. The ChEs of *Eisenia fetida* are different from that of *N. diversicolor* and include at least two types of PrChEs. The AChE activity is located principally in the anterior region of the worm (head) in *N. diversicolor,* whereas the ChE activity of *E. fetida* is located throughout the body. The electrophoretic characterization of *N. diversicolor* and *E. fetida* ChEs showed, respectively, six and two isoforms with disc-PAGE, and three (55,000, 47,000, and 17,000) and five molecular forms (628,000, 301,000, 235,000, 106,000, and 53,800) with PAGGE; substantial activity remained at the top of the PAGGE gel in both species.

Introduction

Cholinesterases (ChEs) are widely distributed in the animal kingdom but have been studied in only a few groups of invertebrates. In mammals, ChEs are typically separated into two major groups, the true cholinesterases (*i.e.,* acetylcholinesterases: AChEs; E.C. 3.1.1.7) and the pseudocholinesterases, namely the butyrylcholinesterases (BuChEs; E.C. 3.1.1.8).

AChEs preferentially hydrolyze acetylcholine as substrate and are very sensitive to eserine inhibition, whereas BuChEs preferentially hydrolyze butyrylcholine, are sensitive to eserine, and are selectively inhibited by iso-OMPA (tetraisopropyl pyrophosphoramide). The ChEs of invertebrates appear to be a group of related enzymes with widely varying properties that are also distinct from those of mammalian ChEs; substrate specificity and patterns of inhibition are especially variable. As a consequence, invertebrate ChEs cannot be separated into AChEs and pseudocholinesterases, as mammalian ChEs are, so the term 'pseudocholinesterase' is irrelevant to invertebrates.

Among annelids, ChEs have been characterized in two species of oligochaetes, *Allobophora caliginosa* (Principato *et al.,* 1978, 1979, 1989; Kaloustian, 1981) and *Eisenia fetida* (Andersen *et al.,* 1978; Stenersen, 1980a, b), and in the freshwater hirudinean *Hirudo medicinalis* (Principato *et al.,* 1981, 1983). But no ChEs have been biochemically characterized from the remaining, and largest, class of annelids, the polychaetes.

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Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; iso-OMPA, tetraisopropyl pyrophosphoramide; LS, low-salt buffer; LST, low-salt/Triton X-100 buffer; S, ChE-soluble fraction; DS, ChE detergent-soluble fraction; DTNB, 5,5'-dithio-(2 nitrobenzoic acid); ATCh, acetylthiocholine iodide; PrTCh, propionylthiocholine iodide; BuTCh, butyrylthiocholine iodide; disc-PAGE, disc polyacrylamide gel electrophoresis; PAGGE, polyacrylamide gradient gel electrophoresis.

Polychaetes are restricted to the marine domain and are considered as the most primitive annelids on the basis of morphology, physiology, and development. Polychaetes are also among the first coelomates and are therefore of special phylogenetic interest.

Here we report the biochemical characterization of a ChE from a polychaete, *Nereis diversicolor;* the results have been compared with those of an oligochaete, *Eisenia fetida.*

Materials and Methods

Animals

The polychaete *N. diversicolor* (O. F. Müller) was harvested at low tide from the estuary of the great coastal river Aa near Grand Fort Philippe in northern France. The oligochaete *E. fetida* (Savigny) was harvested from a living stock that had been bred in the laboratory.

Samples

Tissues were homogenized in one of these following buffers: low-salt buffer (LS): 0.01 *M* Tris/HCl, pH 8.0; or low-salt/Triton X-100 buffer (LST): 1% (v/v) Triton X-100 in 0.01 *M* Tris/HCl, pH 8.0.

Four to six adults, *N. diversicolor* or *E. fetida,* were washed to remove external sediment and then homogenized at 4°C in four volumes of LS buffer. After centrifugation (15,000 × *g,* 30 min, 4°C), the pellet was submitted to a second cycle of homogenization in the same buffer, and the two supernatants were pooled. This final supernatant constitutes the ChE-soluble (S) fraction. The pellet was then incubated twice with four volumes of LST buffer for 1 h at 4°C, which produced a detergent-soluble (DS) extract; *i.e.,* a ChE fraction bound to membranes.

In some experiments, a direct extraction in LST buffer was performed. This is referred to in the text as a "direct DS" extract.

Finally, two parts of the body (head, posterior part) were extracted with LS buffer, as had been done for whole animals. In the case of the electrophoretic analysis, the sample consisted of the first two to four segments (prostomium, peristomium, and first metamers) of 60 worms.

Cholinesterase activity

Protein content and ChE activity were determined according to the method of Bradford (1976) and Ellman *et al.* (1961), respectively; these methods were adapted for measurement with a microplate reader (Dynatech MR 5000). For protein content measurements, 5 or 10 μl of a $\frac{1}{50}$ diluted tissue extract was adjusted to 160 μl with milli-Q water (Millipore), then 40 μl of Bio-Rad protein reagent was added. The absorbance was read at 410 nm, and the protein content of the sample was determined with respect to a γ globulin calibration curve.

Kinetic measurements were performed at 20°C with different thiocholine esters (substrates). Reactions were performed in 300 μl of 0.1 *M* phosphate buffer, pH 8.0, containing (a) 20 μl of 0.01 *M* DTNB (5,5′-dithio-(2 nitrobenzoic acid)), (b) 20 μl of 0.075 *M* substrate, and (c) 10 μl of tissue extracts. DTNB and substrate solutions were prepared in phosphate buffers at pH 7.0 and 8.0, respectively. Thiocholine, a product derived from the enzymatic reaction, combines with DTNB to produce the yellow ion that was measured at 410 nm. The rate of absorbance was linear for at least 2 min, and the slope of each curve was used to evaluate the initial rate of substrate hydrolysis. The enzymatic activity was corrected for spontaneous hydrolysis of the substrate and was expressed as nmol/min/mg of protein.

Substrate specificity and inhibition profile

The rates of hydrolysis of three substrates—acetylthiocholine iodide (ATCh), propionylthiocholine iodide (PrTCh), and butyrylthiocholine iodide (BuTCh)—were measured. Inhibition profiles were determined for the soluble (S) extracts *versus* 10^{-12} to 10^{-3} *M* eserine, or *versus* 10^{-7} to 10^{-3} *M* iso-OMPA (tetraisopropyl pyrophosphoramide); these agents inhibit all ChEs and BuChEs, respectively. The inhibitors, prepared as 0.1 *M* stock solutions in absolute ethanol, were diluted to the appropriate concentration with phosphate buffer for subsequent use. Aliquots (10 μl) of each sample were then incubated for 30 min at room temperature with the inhibitor. The substrate and reactive DTNB were then added, and the cholinesterase activity was measured.

Electrophoretic analysis of ChEs

Disc polyacrylamide gel electrophoresis (disc-PAGE) was carried out on 7.5% homogenous acrylamide slab gels (115 × 100 × 0.75 mm). Tris/HCl buffer (0.375 *M,* pH 8.8) with 15% glycerol was used in the preparation of the separating gel, and Tris/HCl (0.062 *M,* pH 6.8) with 15% glycerol was used in the preparation of the stacking gel (4.5% acrylamide). The running buffer contained 0.025 *M* Tris and 0.192 *M* glycine (pH 8.3). Samples were prepared as follows: to the protein solution was added an equal volume of the sample buffer (0.062 *M* Tris/HCl, pH 6.8, 20% sucrose (w/v) and 0.1% bromophenol blue (w/v)), and 35 μl was applied to each well of the gel. After equilibration of the gel, migration was carried out at 4°C for about 5 h at a constant voltage (200 V); the run was terminated when the marker dye reached 8 cm from the top of the separating gel.

Polyacrylamide gradient gel electrophoresis (PAGGE) was performed on 5%–30% acrylamide gel with the same

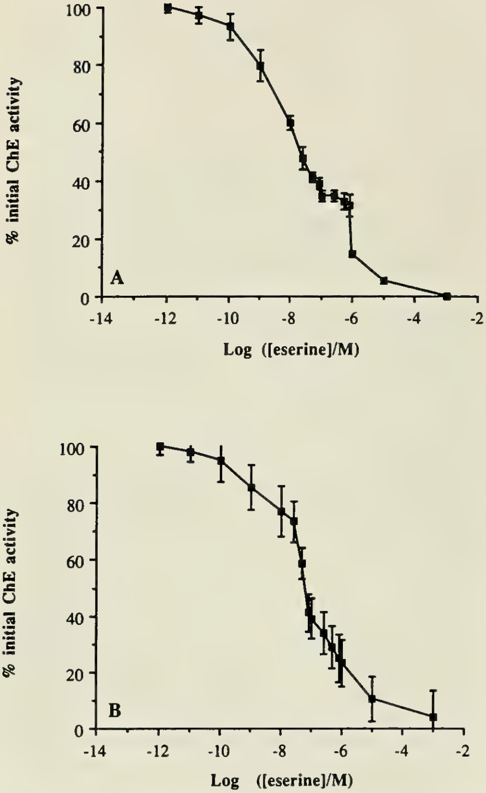

Figure 1. Effect of eserine (10^{-12} M to 10^{-3} M) on acetylthiocholine hydrolysis in the soluble fractions of *Eisenia fetida* (A) and *Nereis diversicolor* (B). Vertical bars denote SD of the mean for three determinations.

buffers and sample preparation as described above for PAGE. Several methods for the histochemical detection of ChEs in polyacrylamide gels were assayed. All were based on the hydrolysis of a thiocholine ester and the formation of an insoluble copper complex (Karnovsky and Roots, 1964; Koelle and Friedenwald, 1949). Original procedures were modified by elevation of the incubation pH to 8.0 in 0.1 M phosphate buffer. ChE isoenzymes were identified through the use of a specific inhibitor of ChEs (10^{-3} M eserine sulfate) and by the comparative hydrolysis of different thiocholine esters (ATCh, PrTCh, and BuTCh). Controls lacking substrate were prepared routinely. Apparent molecular weights were measured with respect to the weights of standard proteins from Sigma (α-lactalbumin from bovine milk, 14,200; chicken egg albumin, 45,000; bovine serum albumin, 66,000 [monomer], 132,000 [dimer]; and jack bean urease, 272,000 [trimer], 545,000 [hexamer]).

Results

ChEs inhibition and substrate specificity

The eserine sensitivity of ATCh splitting activity in a soluble (S) extract of *N. diversicolor* or *E. fetida* is shown in Figure 1. For *E. fetida* (Fig. 1A) the curve is clearly biphasic, indicating the presence of at least two types of ChEs with different eserine sensitivities. The curve obtained for *N. diversicolor* (Fig. 1B) is monophasic, indicating the presence of only one kind of ChE.

With 10^{-3} M iso-OMPA (inhibitor of pseudocholinesterases) and ATCh as substrate, extracts of *E. fetida* were inhibited by 20%, whereas no change was found with extracts of *N. diversicolor,* suggesting that the ChEs of the latter species are AChEs. This result was further confirmed by the study of substrate specificity. In fact, substrates were preferentially hydrolyzed by the ChEs in S extracts of *N. diversicolor* in the following decreasing order: ATCh, PrTCh, and BuTCh (see Fig. 2). Thus the ChEs of *N. diversicolor* are AChEs. By contrast, in the soluble fraction of *E. fetida,* the substrates preferentially hydrolyzed were, in decreasing order, PrTCh, then ATCh, and finally BuTCh (Fig. 2). This result strongly suggests that the ChEs of this species (or at least their most active forms) are PrChEs.

The rate of hydrolysis of S extracts (Table I) with the best substrate for each species was 6.7-fold higher for *E. fetida* than for *N. diversicolor* (24.30 *versus* 3.61 nmol/min/mg protein).

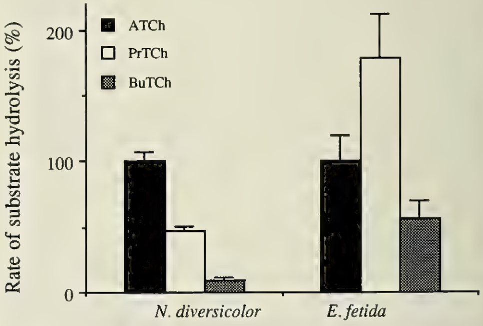

Figure 2. Substrate specificity: preparations of soluble ChEs from *Nereis diversicolor* and *Eisenia fetida* were tested for their ability to hydrolyze different thiocholine esters (ATCh, PrTCh, and BuTCh). Results are expressed as percentage of the controls (ATCh hydrolysis). Vertical bars denote SD of the mean for three determinations.

Table I

Specific ChE activity (nmol/min/mg protein) in soluble fractions of Nereis diversicolor *and* Eisenia fetida *with various substrates (ATCh, PrTCh, and BuTCh)*

	Specific activity (n mol/min/mg protein)		
Species	ATCh	PrTCh	BuTCh
N. diversicolor	3.61 ± 0.27	1.70 ± 0.12	0.32 ± 0.09
E. fetida	13.61 ± 2.60	24.30 ± 4.52	7.63 ± 1.95

Kinetic measurements were performed at 20°C in 300 μl of 0.1 *M* phosphate buffer (pH 8.0) added with 20 μl of 0.01 *M* DTNB, 20 μl of 0.075 *M* substrate (ATCh, PrTCh, or BuTCh), and 10 μl of tissue extracts. The reaction rate was monitored by the increase in absorbance at 410 nm as a function of time. Data are means ± SD.

Table II

Rate of ATCh and BuTCh hydrolysis in soluble fractions of different body regions of Nereis diversicolor *and* Eisenia fetida

	Specific activity (nmol/min/mg protein)	
	ATCh	PrTCh
N. diversicolor		
Head	7.62 ± 0.33	3.38 ± 0.53
Body	2.80 ± 0.93	1.42 ± 0.09
Whole animal	3.61 ± 0.27	1.70 ± 0.12
E. fetida		
Head	13.79 ± 1.90	24.20 ± 2.52
Body	11.57 ± 1.80	21.75 ± 1.02
Whole animal	13.61 ± 2.61	24.30 ± 4.52

Kinetic measurements were performed at 20°C in 300 μl of 0.1 *M* phosphate buffer (pH 8.0) added with 20 μl of 0.01 *M* DTNB, 20 μl of 0.075 *M* substrate (ATCh or PrTCh), and 10 μl of tissue extracts. The reaction rate was monitored by the increase in absorbance at 410 nm as a function of time. Data are means ± SD.

Localization of ChE activity in the body

S extracts from the anterior part (head) of *N. diversicolor* possess a specific activity at least twofold higher than those from the body region (Table II). By contrast, S extracts from the same two parts of *E. fetida* have the same specific activities.

Sequential extraction of ChEs

A similar procedure was used to determine the relative activities of the soluble enzymes (S) and the enzymes bound to membranes (DS). In *N. diversicolor,* most of the AChE activity was recovered from the soluble fraction (95% in S *versus* 5% in DS); and a similar result was obtained for the PrChEs of *E. fetida* (80% in S *versus* 20% in DS) (Fig. 3).

Electrophoretic analysis of ChEs

Based on the localization of ChEs, an electrophoresis study was conducted with S extracts or "direct DS" extracts from *N. diversicolor* heads and from *E. fetida* whole bodies. Neither the original Karnovsky and Roots method nor one modified by increasing the pH to 8.0 revealed any ChE activity in *N. diversicolor* samples. A weak reaction was obtained with the original procedure of Koelle and Friedenwald, and rather good results were obtained when the pH was raised to 8.0. In *E. fetida,* the two methods allowed detection of ChE activity, either with the original pH (5.6) or with the pH increased to 8.0 (data not shown).

Electrophoretic analysis with disc-PAGE showed the ChE activity remaining at the same level, either for freshly prepared samples, or for samples that were stored frozen (−70°C) for several weeks. For each species, the activity pattern for the ChE soluble fraction was the same whether ATCh or PrTCh was used as substrate (Fig. 4A, lanes 1, 2). With BuTCh (Fig. 4A, lane 3), no activity was found for *N. diversicolor,* but with *E. fetida* weak activity was revealed with BuTCh (Fig. 4B, lane 3). When Triton X-100 was added to extract ChEs ("direct DS" extracts) (Fig. 4A, B) no differences appeared. Eserine treatment completely blocked the reaction with both species and with all substrates, attesting to the specificity of the ChE assay used (Fig. 4A, B, lane 5). Disc-PAGE gave six bands of activity in *N. diversicolor;* the first four had a very variable density in repeated experiments, but showed no difference, either with ATCh or with PrTCh as substrate (Fig. 4A). Notice that a substantial amount of ChE activity remained in the sample gel (Fig. 4A). The patterns of distribution of isoenzymes in different parts of the body were similar,

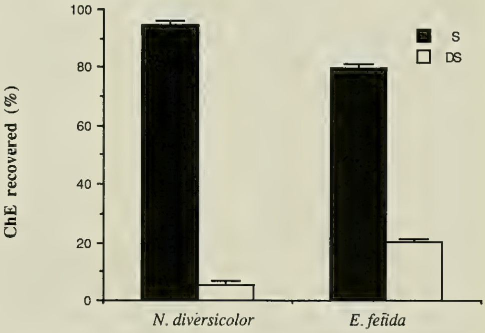

Figure 3. The relative yields of ChEs in soluble (S) and detergent-soluble (DS) fractions from *Nereis diversicolor* and *Eisenia fetida.* Vertical bars denote SD of the mean for three determinations.

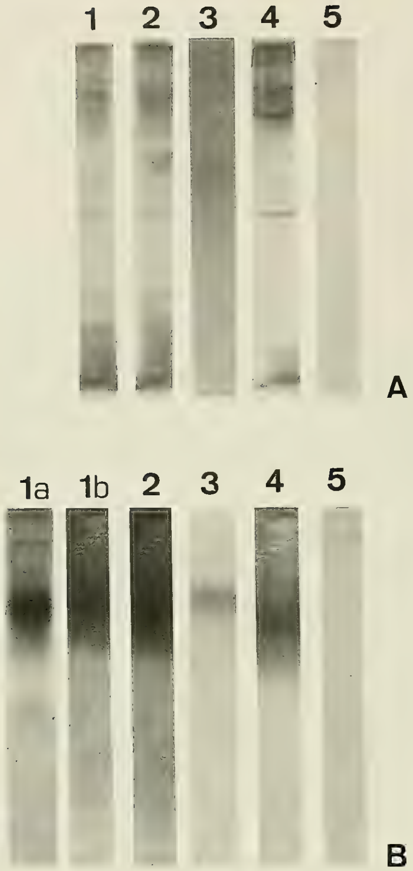

Figure 4. Disc electrophoresis of ChEs from *Nereis diversicolor* (A) and *Eisenia fetida* (B) in 7.5% polyacrylamide gels. Variation in staining reaction: (1) S fraction ATCh hydrolysis; (2) S fraction PrTCh hydrolysis; (3) S fraction BuTCh hydrolysis; (4) DS fraction ATCh hydrolysis; (5) S fraction eserine (10^{-3} *M*) inhibition; (1a and 1b) variability in the intensity of band 1 with S fractions and ATCh hydrolysis.

but not with the same intensity (weaker in the posterior part). Two isoenzymes were found in *E. fetida.* The first had a very variable density and was even absent in repeated experiments (Fig. 4B, lanes 1a, b). The fastest moving band was more intense with PrTCh than with ATCh or BuTCh, which confirms the results with the Ellman procedure. As in *N. diversicolor,* a large amount of activity remained at the top of the gel (Fig. 4B).

PAGGE gave, respectively, three and five bands of activity in *N. diversicolor* and in *E. fetida* (Fig. 5A, B). In both cases, much of the activity remained at the top of the gel. The apparent molecular weights of *N. diversicolor* ChEs were 18,500 ± 3500; 47,000 ± 4500 and 55,000 ± 4400 ($n = 3$). A similar intensity was revealed with ATCh or PrTCh as substrate; no activity was found with BuTCh (Fig. 5A). In *E. fetida,* the apparent molecular weights of the five bands moving into the gel were estimated to be 628,000 ± 19,730 (1); 310,000 ± 5470 (2); 235,000 ± 3200 (3); 106,000 ± 5300 (4); and 53,800 ± 7010 (5) ($n = 5$). The slowest moving band (band 1) had a variable density and was even absent from one experiment to another (Fig. 5B, lanes 1a, b), whereas the other bands developed at a higher intensity with PrTCh than with ATCh or BuTCh as substrate (Fig. 5B, lanes 1b, 2, 3). Bands 2, 3, and 5 were more intense with ATCh than with BuTCh as substrate, whereas band 4 had a higher density with BuTCh than with ATCh.

Discussion

This study demonstrates biochemically, on the basis of both inhibition profiles (eserine and iso-OMPA) and substrate specificity (ATCh, PrTCh, and BuTCh), that an enzyme sharing the properties of AChE is present in the soluble fraction of the nereiid polychaete *N. diversicolor.* Moreover, the measurement of ChE activity with two different techniques (spectrophotometric measurements by the Ellman method, and detection on polyacrylamide gels) lead to the same results. These data confirmed earlier microscopical results of Dhainaut-Courtois (1977) and Dhainaut-Courtois *et al.* (1979a, b) showing ChE activity in different regions of *Nereis pelagica* nervous system (super- and subesophageal ganglia, ventral nerve cord). These authors noticed, in the peripheral and central nervous systems, that the reaction product completely disappeared when the specific inhibitor eserine (10^{-3} *M*) was used, whereas iso-OMPA (10^{-4} *M*), a specific inhibitor of BuChEs, only slightly lowered the intensity of the reaction. That would indicate the existence of a true AChE in the nervous system of *Nereis pelagica.*

We have also found that the cholinesterases of the oligochaete *E. fetida*, including at least two types of PrChEs, are different from those of *Nereis.* These data are consistent with those obtained by Stenersen *et al.* (1992), who also noticed that *E. fetida* has at least two ChEs with different bimolecular inhibition constants for all of the inhibitors (pesticides) tested. Hydrolyzing the best substrate, the specific activity of *E. fetida* PrChE is about sevenfold higher than that of *N. diversicolor* AChE.

Augustinsson (1963) used the term PrChE to distinguish enzymes with a higher activity towards PrTCh than towards other esters. About phylogenetic relationships, ChEs have been found in all invertebrate phyla except for some species of Cnidaria (Scemes *et al.,* 1982). However, in

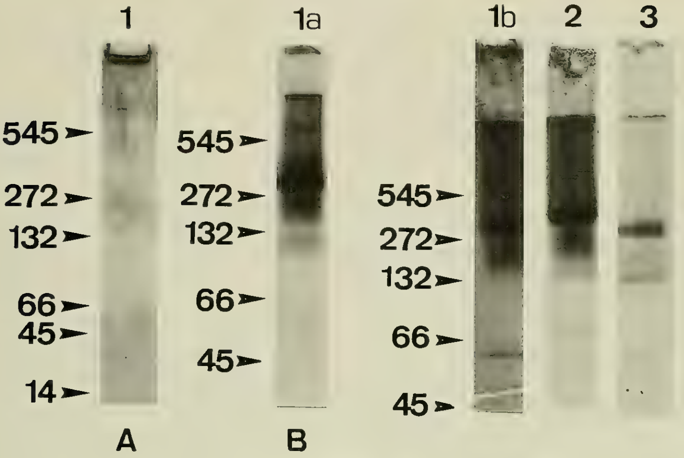

Figure 5. Polyacrylamide gradient gel electrophoresis of ChEs from *Nereis diversicolor* (A) and *Eisenia fetida* (B). (1) S fraction ATCh hydrolysis; (2) S fraction PrTCh hydrolysis; (3) S fraction BuTCh hydrolysis; (1a and 1b) variability in the intensity of band 1 with S fractions. The mobilities of 6 standard proteins run in parallel are indicated with their molecular weight values: α-lactalbumine 14,2000; chicken egg albumin 45,000; bovine serum albumine 66,000 (monomer), 132,000 (dimer); and Jack bean urease 272,000 (trimer), 545,000 (hexamer).

members of the Crustacea (*Squilla mantis,* Principato *et al.,* 1988), Mollusca (*Helix pomatia,* Principato *et al.,* 1984), Sipunculida (*Sipunculus nudus,* Talesa *et al.,* 1993), Oligochaeta (*Allobophora caliginosa,* Principato *et al.,* 1989), and Hirudinea (*Hirudo medicinalis,* Principato *et al.,* 1981, 1983) only PrChE activity was demonstrated. Nevertheless, the physiological function of PrChE is not clearly defined, although PrCh was found in animal tissues (Augustinsson, 1959; Winners *et al.,* 1978). Although these enzymes hydrolyzed PrTCh optimally, Talesa *et al.* (1993) recently proposed to call them AChE to underline their fulfillment of conditions characteristic of AChE (E.C. 3.1.7.7.): they hydrolyzed ATCh significantly, they were inhibited by eserine at concentrations usual for AChEs, and they were inhibited by excess ATCh.

With 7.5% disc-PAGE, six isoforms of ChEs were observed in *N. diversicolor,* and only one or two in *E. fetida.* In both cases, an important zone of ChE activity did not enter the stacking gel; it could correspond to a high molecular weight form of ChEs or an aggregated form appearing in aqueous medium and able to resist the addition of 1% Triton X-100. The results obtained with *E. fetida* are different from those of Stenersen (1980b) who found, on the same species, three different isoforms, but with a polyacrylamide gel concentration of 5%.

Previous works done by Stenersen (1980b) revealed the existence in *E. fetida* of two types of ChEs called E_1 and E_2 by this author. E_1 was a PrChE, whereas E_2 was a nonspecific ChE. E_1 would be represented by four molecular weight forms of about 65,000, 105,000, 235,000, and 312,000. E_2 was not further characterized. In fact, because of its high molecular weight, it stayed in the stacking gel. By contrast, Andersen *et al.* (1978) found only one PrchE, with a molecular weight of about 108,000, in the same species. In our study, components of 53,800, 106,000, 235,000, and 310,000—and a very high molecular weight form that stayed in the stacking gel—were observed and confirm the results obtained by Stenersen (1980b). An additional component of 628,000 was sometimes observed. According to Stenersen, the high molecular weight forms of ChEs probably result from an assemblage of unstable active monomers of 65,000. The molecular assemblage of PrChEs has also been studied in other Annelida. In the oligochaete *Allobophora caliginosa,* Principato *et al.* (1989) showed that the animals contain one PrChE of 180,000, which could be a tetramer of two molecular weight subunits of 28,000 and 62,000. In the leech *Hirudo medicinalis,* the molecular weight of PrChE is higher (*i.e.,* 224,000) than that of *A. caliginosa* (Principato *et al.,* 1981). And in the coelomate unsegmented marine worm *Sipunculus nudus,* closely related to polychaete worms, the PrChE has a molecular weight of 135,000, which results from the assemblage of two identical subunits of 67,000 (Talesa *et al.,* 1993). Results obtained in *N. di-*

versicolor clearly indicate that the AChE is represented by two components with similar molecular weights (*i.e.*, 47,000 and 55,000), a third component with a lower molecular weight (18,000), and a very important fraction that does not enter the gel. This result is particularly important because at this time there is no information about AChE in the polychaetes.

Acknowledgments

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Cement Proteins of the Acorn Barnacle, *Megabalanus rosa*

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Abstract. Components of the proteinaceous cement secreted by barnacles have yet to be studied because of their insolubility. We solubilized and characterized the proteins of secondary cement, which is produced when the barnacle is detached from the substratum, in *Megabalanus rosa.* The cement was fractionated, according to its solubility in aqueous formic acid, into a soluble fraction, SF1 (21%); a fraction soluble after reduction, SF2 (37%); and a fraction insoluble after reduction, IF (42%). Analysis of the SF1 and SF2 by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) revealed that they contained three polypeptides (SF1-60 k, -57 k, -47 k) and one polypeptide (SF2-60 k), respectively. The amino acid compositions of these polypeptides were similar and their *N*-terminal amino acid sequences were identical. These polypeptides had an unusual amino acid composition, rich in Ser, Thr, Ala, and Gly, like the tube cement of a marine polychaete, *Phragmatopoma californica.* The IF, solubilized in aqueous formic acid after cleavage with cyanogen bromide, was shown by SDS-PAGE to contain eight fragment peptides (CB-peptides). *N*-terminal amino acid sequences of the CB-peptides were also determined.

We conclude that the barnacle cement is composed of at least two types of protein: highly hydroxylated protein in the SF1 and SF2 and insoluble protein in the IF.

The SDS-PAGE pattern of CB-peptides from the secondary cement was identical to that of the primary cement produced while the barnacle is attached to a substratum. In addition, immunoblot analysis, using a polyclonal antibody against one of the CB-peptides from the secondary cement, also cross-reacted with a CNBr-fragment peptide of the primary cement. These results indicate that the primary and secondary cements are similar in protein composition.

Introduction

Barnacle cement plays a major role in attaching the animal to various substrata (Walker, 1972). Barnacle cements are classified into two types according to the method of collection: a primary cement produced while the animal is on a substratum, and a secondary cement secreted while the animal is free from a substratum (Saroyan *et al.,* 1970). The two forms of cement are similar in their whole amino acid composition (Naldrett, 1993). Reattachment of barnacles to a new substratum by secondary cement has also been reported (Saroyan *et al.,* 1970; Dougherty, 1990). These reports indicate that the primary and secondary cements are essentially the same (Naldrett, 1993), although there has hitherto been no biochemical evidence to establish this relatedness.

Although amino acid compositions of several barnacle cements have been reported (Walker, 1972; Naldrett, 1993), detailed analysis of the components of the cement has been thwarted by their insolubility. In the study of such adhesive proteins, we generally face two obstacles. First, it is difficult to measure adhesive activity during purification. Second, it is difficult to purify and analyze the adhesive proteins after secretion because they are usually insolubilized in the adhesive process (Waite, 1987).

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Abbreviations: SF1 and SF2, soluble fraction 1 and 2 of the cement; IF, formic-acid-insoluble fraction of the cement; CB-peptides, major peptides derived from IF using CNBr treatment; DOPA, 3,4-dihydroxyphenylalanine; CNBr, cyanogen bromide; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; TRICINE, *N*-Tris(hydroxymethyl)methylglycine; PVDF, polyvinylidene difluoride; CAPS, 3-cyclohexylaminopropanesulfonic acid; NBD-F, 4-fluoro-7-nitrobenzofurazan; RP-HPLC, reversed-phase-high-performance liquid chromatography; Hyp, hydroxyproline; EDTA, ethylenediaminetetraacetic acid.

Table I

Amino acid composition (residues per thousand) of cement from Megabalanus rosa; *composition of cement from two species of* Balanus *(Walker, 1972) is given for comparison*

Amino acid	*M. rosa*	*Balanus crenatus*	*Balanus hameri*
AsX	90.66	82.81	75.15
GlX	91.49	86.35	87.02
Ser	99.05	76.91	109.29
Gly	79.20	85.92	79.50
His	13.29	21.61	21.90
Thr	70.49	62.27	63.13
Ala	74.73	64.67	66.11
Arg	56.02	61.35	56.30
Pro	49.16	60.56	80.66
Val	72.51	21.86	26.39
Met	16.00	6.74	6.89
Cys	16.03	72.83	65.52
Ile	53.01	53.42	42.56
Leu	82.76	81.11	84.43
Trp	0.00	0.00	0.00
Phe	37.09	39.85	35.29
Lys	56.66	67.92	52.59
Tyr	41.84	53.80	47.28

The first underwater adhesive protein purified from a marine invertebrate was a byssus protein of a marine mussel, *Mytilus edulis* (Waite and Tanzer, 1981; Waite, 1992a; Laursen, 1992), which contained peptidyl 3,4-dihydroxyphenylalanine (DOPA). Since then, similar DOPA-containing adhesive proteins from other mussels (Rzepecki *et al.*, 1991) and a marine polychaete (Waite, 1992b; Waite *et al.*, 1992) have been purified by using DOPA as a marker. However, other underwater adhesive proteins—including barnacle cement—that lack a marker such as DOPA have not yet been purified and characterized.

In the present study, we fractionated the proteins in barnacle cement according to solubility, and purified and characterized the major proteins in these fractions. Biochemical evidence for the identity between primary and secondary cements was also examined.

Materials and Methods

Chemicals

All chemicals were of the highest grade, and most were purchased from Wako Pure Chemicals (Osaka).

Specimens

Acorn barnacles, *Megabalanus rosa,* were collected from a floating buoy in Miyako Bay, Iwate Prefecture, Japan. Detached barnacles were used for collection of the cements.

Cement collection

The primary cement was collected by scraping the lower surface of the calcareous base immediately after dislodgement of the barnacle.

To avoid contamination during collection of the secondary cement, the calcareous base of the barnacle was covered with Parafilm (American National Can Co.) after the animal was detached from the substratum. The barnacle was then immersed in aerated seawater in an aquarium. Secondary cement secreted on the base of barnacle was collected with a microspatula on the following day. The collected cement was dialyzed against pure water at 4°C, lyophilized, and stored at −20°C until used.

Fractionation by centrifugation

The stored secondary cement was suspended in 80% formic acid, diluted with ultrapure water to 10% formic acid, and centrifuged at 200,000 × *g* for 1 h. The supernatant was designated as soluble fraction 1 (SF1). The precipitate was resuspended in 10% formic acid, and this centrifugation was repeated twice more to separate the SF1 from the precipitate. The protein content remaining in the supernatant after the third centrifugation was negligible. Cys residues of the precipitate were reduced and *S*-alkylated with 4-vinylpyridine in the presence of tri-*n*-butyl phosphine in the dark at room temperature overnight (Rüegg and Rudinger, 1977), and excess chemicals were then removed by dialysis against ultrapure water in the dark. The resultant suspension was centrifuged as described above. The precipitate was named the formic-acid-insoluble fraction (IF), and the supernatant was designated as soluble fraction 2 (SF2). After evaporation of the formic acid, each fraction was lyophilized.

Solubilization of the formic-acid-insoluble fraction

In order to solubilize the IF, it was suspended in 1% cyanogen bromide (CNBr) in 70% formic acid and agitated in the dark at room temperature for 24 h. After evaporation of the CNBr, the sample was dissolved in 80% formic acid, diluted with water to 10% formic acid, and centrifuged. The supernatant was lyophilized. Most of the IF was solubilized by this treatment, and the residual precipitate was negligible.

Electrophoretic analysis and electroblotting

With one exception samples for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were incubated at 100°C for 3 min in the sampling buffer containing 2% SDS and 5% 2-mercaptoethanol: The nonreduced sample of SF1 was incubated in the same conditions except that the buffer lacked 2-mercaptoethanol. The samples were then applied to discontinuous SDS-PAGE

Table II

Amino acid composition (residues per thousand) of each fraction in the cement from Megabalanus rosa; *composition of whole cement from* M. rosa *(this study) and the tube worm* Phragmatopoma californica *(Jensen and Morse, 1983) is given for comparison*

	Fraction*					Whole cement	
Amino acid	IF	SF1-60k	SF1-57k	SF1-47k	SF2-60k	*M. rosa*	Tube worm
AsX	79.88	85.74	86.51	85.22	87.51	90.66	20.00
GlX	90.43	87.19	86.82	78.68	86.50	91.49	9.00
Ser	89.04	(139.46)	(152.63)	(132.04)	(159.81)	99.05	290.00
Gly	63.28	153.24	159.76	142.10	147.84	79.20	240.00
His	14.02	4.70	4.38	11.40	2.48	13.29	28.00
Thr	53.76	(129.37)	(127.38)	(114.61)	(138.06)	70.49	0.00
Ala	62.40	124.17	125.57	115.18	124.10	74.73	65.00
Arg	61.73	27.39	28.68	26.28	36.23	56.02	40.00
Pro	54.08	34.19	23.98	29.70	25.45	49.16	24.00
Val	73.86	84.52	78.25	79.78	72.84	72.51	44.00
Met	19.84	0.00	0.00	0.00	0.00	16.00	3.00
Cys	14.04	6.53	6.10	7.61	8.91	16.03	0.00
Ile	63.26	(31.61)	(34.21)	(46.09)	(17.27)	53.01	11.00
Leu	100.15	(35.06)	(28.07)	(52.14)	(17.83)	82.76	33.00
Trp	0.00	ND	ND	ND	ND	0.00	ND
Phe	45.18	10.59	8.45	20.39	6.50	37.09	16.00
Lys	53.66	32.22	31.59	25.46	51.02	56.66	118.00
Tyr	61.41	13.52	17.61	33.32	5.27	41.84	33.00
DOPA	0.00	0.00	0.00	0.00	0.00	0.00	26.00

* In the four fractions of SF1 and SF2, Trp contents were not determined (ND) and Ser, Thr, Ile, and Leu contents (values in parentheses) were determined only by values of 24-h hydrolysates.

according to the method of Laemmli (1970). The *N*-Tris(hydroxymethyl)methylglycine (TRICINE)–SDS-PAGE system (Schägger and Jagow, 1987) was used for separation of fragment peptides (CB-peptides) released from the IF after cleavage with CNBr. Electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Pro Blott, Applied Biosystems) was carried out according to Matsudaira (1987); the 3-cyclohexylaminopropane-sulfonic acid (CAPS) buffer system was used for amino acid analysis and *N*-terminal amino acid sequencing.

Amino acid analysis

Samples for amino acid analysis were hydrolyzed *in vacuo* in constant-boiling HCl (5.7 *N*) including 0.02% phenol or in 4 *M* methane sulfonic acid (Pierce Chemicals) at 110°C for 24 h, 48 h, and 72 h. Cys was detected as cysteic acid after performic acid oxidation (Hirs, 1967). Amino acid compositions of the hydrolysates were determined with a TOSOH automatic amino acid analyzer (TOSOH Corp., Tokyo) using 4-fluoro-7-nitrobenzofurazan (NBD-F) (Dojindo Laboratories) for detection (Miyano *et al.*, 1985) and reversed-phase–high-performance liquid chromatography (RP-HPLC) (ODS-80TM, TOSOH Corp.) for separation.

Protein content was estimated from amino acid analysis of the lyophilized sample, including *n*-Leu (Pierce Chemicals) as an internal standard.

N-terminal amino acid sequence analysis and secondary structure prediction

Peptide bands transferred to a PVDF membrane were excised after visualization with Coomassie brilliant blue R-250 and analyzed with a pulse liquid-phase sequencer (473A, Applied Biosystems). Secondary structure prediction from a partial sequence of CB-peptides was carried out with a GENETYX-MAC Version 7.0 (Software Development Co., Tokyo) as described by Chou and Fasman (1978).

Preparation of the antibody and immunoblot analysis

Polyclonal antibodies against CB-2 peptide were raised in rabbits by serial subcutaneous injections of the electrophoretically isolated CB-2 peptide in Freund's complete adjuvant. Specificity of the antibody was studied by immunoblotting with the CB-peptides, as described elsewhere (Enami *et al.*, 1991).

Results

The protein content of the dialyzed secondary cement was estimated to be more than 90% of dry weight by the amino acid analysis. Weight percentages of SF1, SF2, and IF in the cement were estimated to be 21%, 37%, and 42%, respectively. Table I shows the amino acid com-

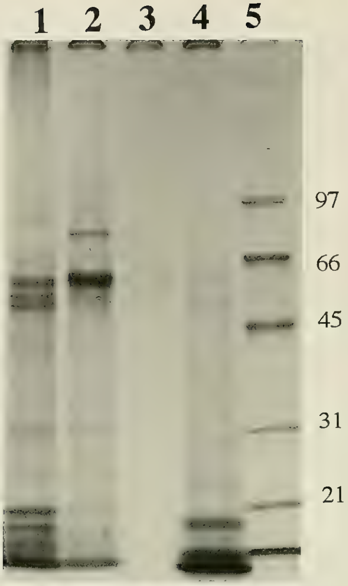

Figure 1. (A) SDS-PAGE pattern for each fraction in cement. Lanes 1–3: SF-1, SF-2, and IF each prepared from 100 μg of cement. Lane 4: CB-peptides. Lane 5: low molecular weight standards (BIO-RAD). Samples were applied to SDS-PAGE (gradient, 8%–16% T) and gel was stained with Coomassie blue R-250. Numbers on side of lane 5 indicate molecular masses (kDa).

position of the *M. rosa* cement in comparison with the cements of other barnacles. Although the Cys content was lower in *M. rosa* than in other species, the profiles were similar. Modified amino acids such as hydroxyproline (Hyp), DOPA, and methionine sulfoxide were not detected in the hydrolysate of the *M. rosa* cement.

SDS-PAGE showed that the SF1 contained three major polypeptides near 60 kDa (Fig. 1). They were named SF1-60 k, -57 k, and -47 k. Some bands with molecular weights less than 20 kDa were also detected in SDS-PAGE of the SF1. The SF2 contained one polypeptide near 60 kDa; it was designated as SF2-60 k. In contrast, the IF gave no detectable bands because of its insolubility in the sampling buffer.

The amino acid compositions of SF1-60 k, -57 k, -47 k, and SF2-60 k were similar (Table II). SF1-60 k, -57 k, -47 k, and SF2-60 k had the same *N*-terminal amino acid sequence: Gly-Lys-Ala-Val-Thr-Val-Gly-Thr-Asp-.

SDS-PAGE of SF1 in the absence of 2-mercaptoethanol showed a single band near 180 kDa but no band near 60 kDa (Fig. 2), suggesting that SF1-60 k, SF1-57 k, and SF1-47 k form an oligomer.

The IF could not be solubilized without cleavage of peptide bonds in any of the solutions tested—7 *M* guanidinium hydrochloride, 8 *M* urea, 5% SDS, 1% Triton X-100, 80% formic acid, 0.1 *M* acetic acid, 1% perchloric acid, 0.5 *M* lithium chloride, or 0.1 *M* ethylenediaminetetraacetic acid (EDTA). Most of the IF was solubilized by the CNBr treatment. The solubilized IF was shown to contain 8 CB-peptides (CB-1 to 8) in SDS-PAGE (Fig. 3). The IF was solubilized by CNBr treatment even though disulfide bonds were not cleaved.

Although no consensus sequence was found in the CB-peptides (Fig. 4), in CB-8 peptide, Leu appeared at every one to four amino acid sites among which hydrophilic amino acids were interspersed. Secondary structure prediction indicated abundance of the β-sheet in CB-peptides, especially CB-1 (84%), CB-3 (80%), CB-4 (83%), CB-6 (77%), CB-7 (63%), and CB-8 (66%).

After CNBr treatment, the primary cement gave the

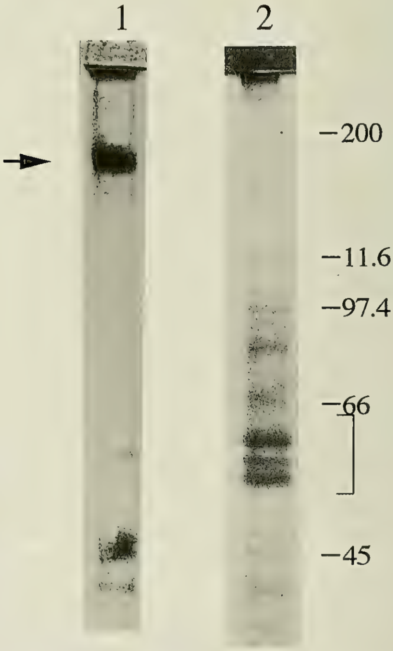

Figure 2. SDS-PAGE pattern of SF1 with or without reductant. Lanes 1 and 2 correspond to SF1 without and with reductant (2-mercaptoethanol) respectively. Arrow in lane 1 shows 180-kDa band, and bracket in lane 2 shows SF1-60 k, SF1-57 k, and SF1-47 k polypeptides. Numbers on side of lane 2 indicate molecular masses (kDa) of low molecular weight standards (BIO-RAD).

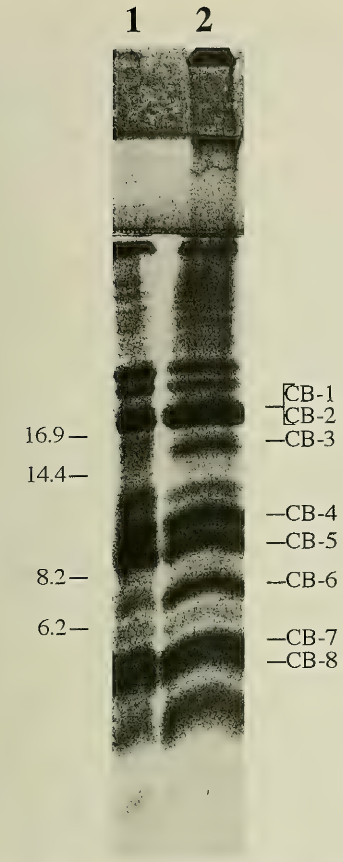

Figure 3. Complete separation of CB-peptides by SDS-PAGE. Lane 1: primary cement treated with CNBr. Lane 2: CB-peptides resolved by TRICINE buffer system (separating gel; 16.5% T, 6% C, 6 *M* urea) with spacer gel (10% T, 3% C). Names of CB-peptides (CB-1 to CB-8) indicated on the right side. Numbers on left side indicate molecular masses (kDa) estimated from molecular weight peptide standards (Pharmacia LKB). Complete resolution of CB-1 and CB-2 achieved using separating gel (16.5% T, 3% C) without spacer gel and urea. Apparent molecular masses of the CB-peptides were as follows: CB-1, 18.8 kDa; CB-2, 18.2 kDa; CB-3, 15.1 kDa; CB-4, 9.6 kDa; CB-5, 8.1 kDa; CB-6, 6.4 kDa; CB-7, 4.6 kDa; CB-8, 4.2 kDa.

same SDS-PAGE pattern as the secondary cement (Fig. 3). The anti–CB-2 antibody reacted with a band of the CNBr-fragment of the primary cement that had the same mobility as CB-2 (Fig. 5). No CNBr-fragment derived from the SF1 or the SF2 reacted with the anti–CB-2 antibody (data not shown).

Discussion

The cement of *M. rosa* was composed of proteins, as reported in other *Balanus* species (Walker, 1972). The amino acid composition of the cement of *M. rosa* was similar to those of two other *Balanus* species, except for the lower Cys content in *M. rosa.* Cements of pedunculate cirripedes, *Lepas anatifera* (Walker and Youngson, 1975) and *L. fascicularis* (Barnes and Blackstock, 1976), were also reported to have lower Cys contents.

The barnacle cement of *M. rosa* yielded three fractions, SF1, SF2, and IF. The absence of cross-reaction of anti–CB-2 antibody with the SF1 and SF2 indicates that the IF is distinguished from the soluble SF1 and SF2. The SF1 and SF2 comprised polypeptides similar in molecular weight, amino acid composition, and *N*-terminal sequence. Although the SF2 was solubilized in aqueous formic acid by cleavage of disulfide bonds, it is not clear whether the polypeptides in the SF2 bind to the IF by a disulfide bond. The SF2 may be merely entangled with the IF without covalent binding. Cleavage of the disulfide bond may facilitate solubility in formic acid. The SF1-60 k, SF1-57 k, and SF1-47 k polypeptides formed an 180-kDa oligomer cross-linked by intermolecular disulfide bonds. Intermolecular disulfide bonds may play a role in the insolubility of these polypeptides in seawater.

The SF1-60 k, SF1-57 k, SF1-47 k, and SF2-60 k polypeptides were highly hydroxylated. In these four polypeptides, amino acids with hydroxyl side-chains accounted for 30% of the total (Ser = 16% and Thr = 14%). The short-side-chain amino acids made up 43% of the total residues (Ser = 16%, Gly = 15%, and Ala = 12%). These unique amino acid compositions are similar to that of the Ser-rich protein in the tube cement of a marine polychaete (*Phragmatopoma californica*), and to sericin, a sticky outer covering of the silk fiber of silkworms, (*Bombyx mori*) (Jensen and Morse, 1988). In the byssus protein of a mussel (*Mytilis*), high contents of the hydoxylated amino acids Ser, Thr, and Hyp (Waite, 1987) and dihydroxyproline (Taylor *et al.*, 1994) have also been reported. Waite suggested that the hydroxyl groups of *Mytilus* byssus

CB-1 : ITIFRGWGLPKLYQPRYTLXGIVXYIKV
CB-2 : QRVAFELAKRFEGLKEPSFRLPLLK
CB-3 : ATYELFIYHSTIYFRSSCAY
CB-4 : SLGGVVAYLQLANIQQAVFISRI
CB-5 : HYESLSDISLKAIFRNKLLNNFPEEVPATRDGV
CB-6 : NRYVYQSIISYTQTVRKPNYYSAEFFRILVE
CB-7 : RNIRSIVAHTILILRARYSITQNNCYGHLTKYYNGIPINA
CB-8 : LRQLIVTLDYLDHELPVILDYEELIAVRLALKKKFDTSVDIFKNRYQLAI

Figure 4. *N*-terminal amino acid sequences of CB-peptides. Cys was detected as pyridylethyl-Cys. X: not determined.

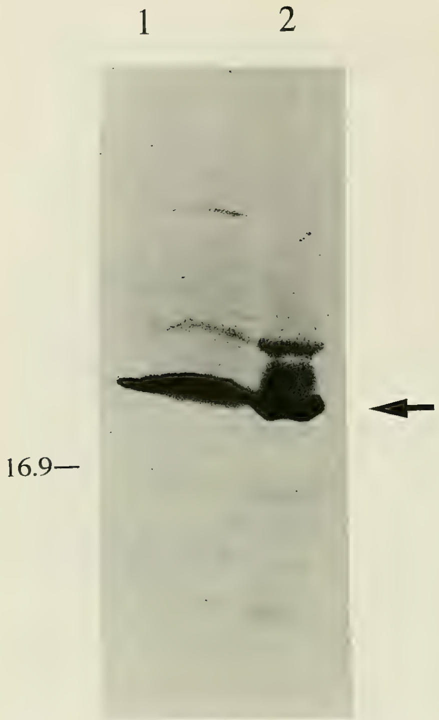

Figure 5. Immunoblotting analysis using polyclonal antibody against CB-2. Lane 1: CB-peptides. Lane 2: primary cement treated with CNBr. Number on side of lane 1 indicates molecular mass (kDa) of molecular weight peptide standards (Pharmacia LKB).

protein play a role in removing the weak boundary-water layer and in spreading the protein onto the surface of the substratum in the process of adhesion. The hydroxylated side-chains of proteins in SF1 and SF2 may be similarly important for underwater adhesion.

The IF was difficult to solubilize in aqueous formic acid without cleavage of peptide bonds. Insolubilization is thought to be an essential feature of the underwater adhesive process. How is the IF protein rendered insoluble? DOPA, reported to be present in *Mytilus* byssus protein (Waite and Tanzer, 1981), may confer insolubility on the protein by making intermolecular cross-linkages between its oxidation product DOPA-quinone and Lys, as suggested by Waite (1987). In this study, as in other research on barnacle cements (Naldrett, 1993), no DOPA was detected in the hydrolysate of *M. rosa* cement: Thus DOPA-quinone cross-linkage does not seem to be responsible for the insolubility of the IF in barnacle cement. Cleavage of Met-X bonds by CNBr promoted solubilization of the IF, although the existence of other intermolecular cross-linkages cleaved by CNBr treatment is not ruled out. Intermolecular cross-linkages are not always necessary for protein insolubility. For instance, the 16-residue peptide $(AlaGluAlaGluAlaLysAlaLys)_2$ spontaneously assembles to form an irreversibly insoluble membrane upon the addition of salt (Zhang *et al.,* 1993). The stability of the membrane is attributed to intermolecular hydrophobic interactions and to ionic bonds of the β-sheet peptide. In silk fibroin, a β-sheet structure contributes to the assembly of monomer fibroin to insoluble fibers (Hamodrakas *et al.,* 1982). The potential abundance of the β-sheet in CB-peptide is consistent with the insolubility of the IF.

SDS-PAGE and immunoblot analysis with an anti–CB-2 antibody indicated that the primary and secondary cements are similar in protein composition. The secondary cement acts at the interface between the calcareous base and the substratum to which the barnacle attaches.

The cement, composed of at least two distinct groups of proteins—formic-acid-insoluble IF and highly hydoxylated proteins in the SF1 and SF2—serves as an underwater adhesive. Cloning of cDNAs corresponding to the IF and the highly hydroxylated proteins in SF1 and SF2 is now in progress.

Acknowledgments

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DNA-Synthesizing Cells in the Heart of *Ascidia obliqua* (Tunicata)

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Abstract. In vertebrate cardiomyocytes, DNA synthesis and mitosis occur in the presence of myofibrils. The myocardium of the adult vertebrates has no cambial elements. We have studied the ultrastructural features and replicative abilities of the heart cells of *Ascidia obliqua,* a representative of the tunicates, which are considered to be closely related to the immediate precursors of vertebrates. The ascidian heart is composed of a pericardium and a myocardium. The pericardium consists of mononucleated cells with well-developed Golgi complexes and expanded channels of rough endoplasmic reticulum. The myocardium consists of mono- and binucleated myoepithelial cells, whose polarity is recognizable in basement lamina underlying the basal surface; centrioles, cilia, and a well-developed Golgi complex located at the apical side; and cross-striated myofibers, one per cell, running along the basal membrane. Four hours after injection of tritiated thymidine ([^{3}H]Tdr), labeled nuclei were found both in pericardial and myocardial cells. Electron microscope autoradiographs show that the [^{3}H]Tdr-labeled nuclei in the myocardium belong to myofiber-containing cells. We failed to observe mitosis; however, the occurrence of centrioles and the high number of binucleated myocytes testify to the ability of these cells to undergo karyokinesis. Large numbers of [^{3}H]Tdr-labeled and prophasic nuclei have been observed in the raphe region, the site of transition of the pericardium into myocardium. The morphological features, such as loss of labyrinth junctions and the acquisition of gap junctions, that distinguish the cells of the raphe region from ordinary pericardial cells give evidence of their premyocytic nature. The resemblance of ascidian myocardium to the vertebrate embryonic one and the presence of cambial zone in the ascidian heart are discussed.

Introduction

In the vertebrates, two types of cross-striated muscle are distinguished on the basis of the interrelationship between the proliferative and differentiative processes that are peculiar to their myocytes. These types are cardiac and skeletal muscles. The former is characterized by a number of peculiarities in ultrastructure (Abdelmeguid and Sorour, 1992) and by the persistence of DNA synthesis and mitoses in myocytes (Rumyantsev, 1991, for review). With the aid of electron microscopy and electron-microscopic autoradiography, DNA synthesis and mitoses have been shown in the human (Schmid and Pfitzer, 1985), mammalian (Langes *et al.,* 1983), avian (Manasek, 1973), and amphibian (Oberpriller and Oberpriller, 1991) myocardial cells. For the fish heart, the data available are limited to a description of mitoses at the light-microscopic level (Kolosova and Nesterenko, 1981) and indirect evidence, from morphometry and DNA content analysis, in favor of hyperplasia (Farrel *et al.,* 1988). Data on the cell mechanisms of heart muscle growth in cyclostomes, cephalochordates, and invertebrate deuterostomes are lacking. Studies of cardiac muscle at these earlier stages of evolution are important for our understanding of both the origin of this type of muscle in evolution and the specific features that make cardiac muscle different from skeletal muscle.

The purpose of the present study was to find out the type of the ascidian myocardium and the extent to which it is similar to the vertebrate cardiac muscle. Ultrastructural and reproductive traits of cells from different parts of ascidian heart—pericardium, myocardium, and raphe

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region—have been investigated. We report here that differentiated cardiomyocytes of the ascidian heart, like those of higher chordates, retain the capacity for DNA synthesis. Unlike the myocardium of vertebrates, however, the ascidian myocardium possesses a permanent cambial zone in the raphe regions.

Materials and Methods

Live specimens of the solitary tunicate *Ascidia obliqua* Alder, 1863, were supplied by the Institute of Marine Biology at the University of Bergen. The animals, ranging in weight from 8 to 12 g, were kept in an aquarium with seawater at about 15°C. Tritiated thymidine (State Institute for Applied Chemistry, St. Petersburg, Russia) with a specific activity of 16 Ci/mmole was injected into the body cavity of the animal at a dose of 10 mCi per gram body weight. The hearts were fixed 4 h later in a modified version of Karnovsky fixative, in which distilled water had been replaced by Ringer solution (Nylund, 1985). After fixation for 3.5 h, the tissue was washed in a phosphate-buffered solution of 8% sucrose and postfixed for 1.5 h in a phosphate-buffered 1% solution of osmium tetroxide. Dehydration through a graded series of alcohols and acetone followed. Finally the tissues were embedded in Epon 812.

For electron microscopy (EM), thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined under a transmission microscope (JEM 7A, Japan). EM autoradiographs were prepared by the method of Larra and Droz (1970), with an exposure time of 60 days.

The share of [^{3}H]Tdr-labeled nuclei (from the total number of nuclei) was calculated from autoradiographs of semithin sections (1 mm thick) stained with methylene blue and azure II after 30 days of exposure to photoemulsion. For each of six animals, 20 sections were examined. The number of counted nuclei averaged 5000 in the myocardium, 3500 in the pericardium, and 800 in the raphe region. The standard error of the means was calculated.

Results

Light-microscopic examination of sectioned material revealed that the heart of *Ascidia obliqua* consists of a single-layered pericardium and a single-layered myocardium that invaginates from the pericardium. The myocardium makes up a V-shaped curve, and hence two myocardial layers can be seen on the transverse sections of the heart (Fig. 1). The flaps of the myocardium are joined together at the raphe by some amorphous noncellular material. Thus, the pericardial and myocardial (heart lumen) cavities are formed.

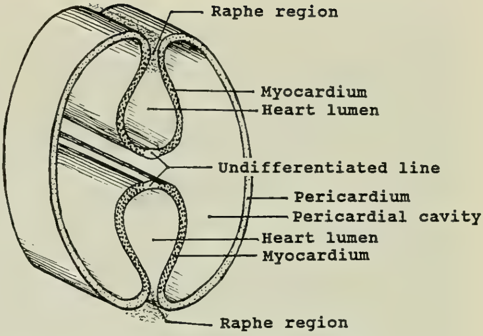

Figure 1. Schematic representation of a short segment of the tubular heart-pericardial complex of *Ascidia obliqua*. Owing to the V-shaped curve of the myocardium, two myocardial layers are seen. In the center of the myocardial layers is an undifferentiated line. The pericardium and myocardium are joined at the raphe.

Pericardium

Pericardial cells are cubiform and resemble cells of secretory epithelium (Fig. 2). Signs of polarity in the structural organization of these cells are recognizable in the basement lamina that underlies their basal surface; the centrioles and cilia located at the apical surface that faces the pericardial cavity; and the well-developed Golgi complex that is always located in the perinuclear cytoplasm near the apical side. A single nucleus lies in the central core of each cell. The nuclei are roundish in shape and about 4 mm in diameter, with a single conspicuous nucleolus. No myofilaments can be found in these cells. Mitochondria containing intramitochondrial granules are present throughout the cytoplasm. A significant portion of the cytoplasm is occupied by very expanded channels of rough endoplasmic reticulum. Some free ribosomes, large numbers of glycogen particles, and enormous autophagosomes are also seen. Intercellular contacts include apical junctional complexes and labyrinth junctions (Figs. 2, 3).

An autoradiographic study showed that some pericardial cells incorporated [^{3}H]Tdr. As seen in light-microscopic autographs, the number of labeled nuclei in the pericardium was as low as 0 to 2 per section and constituted up to 0.4% ± 0.1% of total number of nuclei (see Table I). We failed to observe any mitotic figures in the ordinary pericardial cells.

Raphe region

The cells in the raphe region are closely adjacent to the myocardium. They are distinct from myocardial cells in

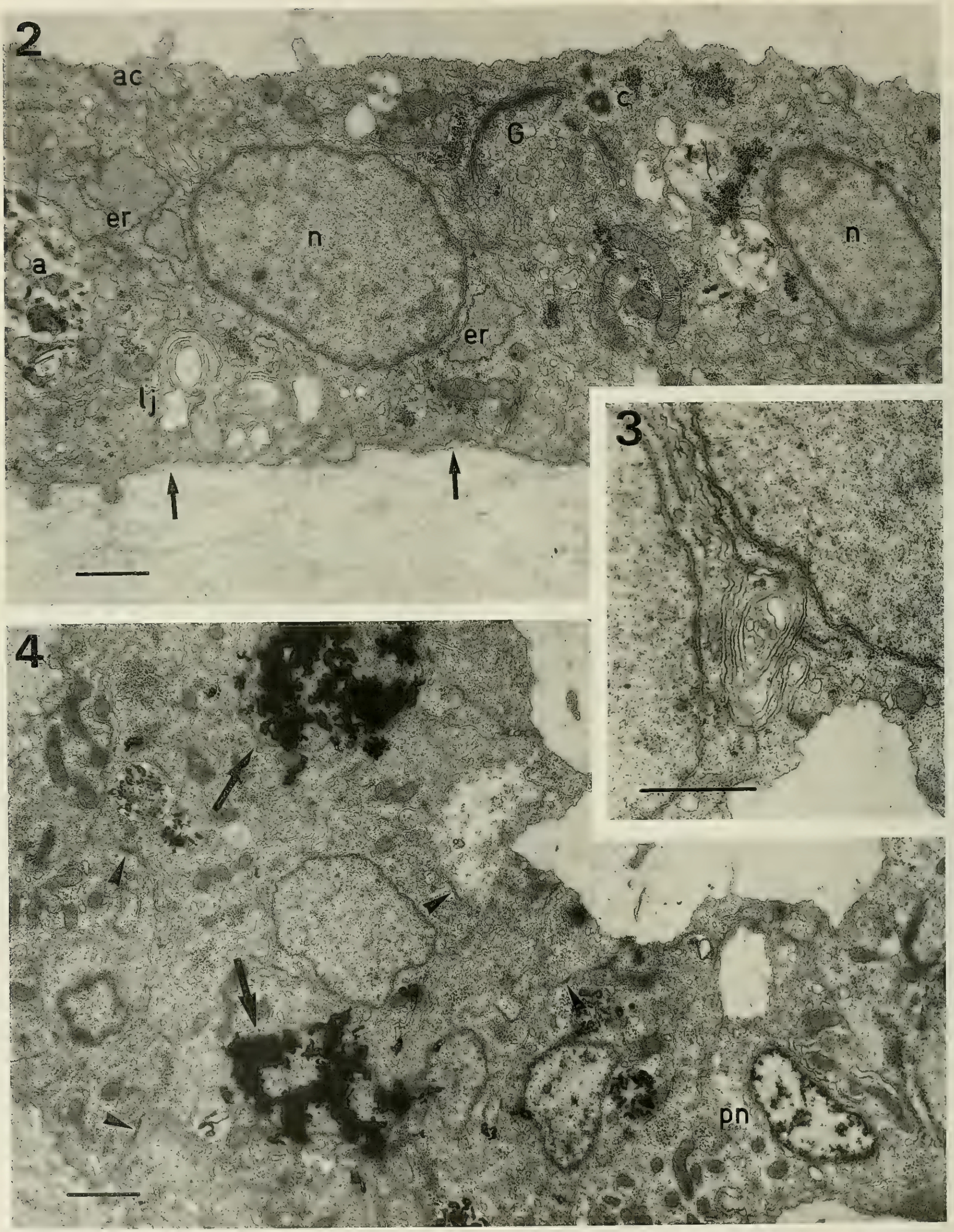
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Table I

Number of labeled nuclei in different compartments of ascidian hearts 4 h after [^{3}H]Tdr injection

Animal	Pericardium	Raphe region	Myocardium
1	8 (3550)	18 (800)	21 (4900)
2	18 (3550)	14 (850)	17 (4950)
3	19 (3400)	4 (850)	31 (5100)
4	7 (3300)	9 (700)	16 (5050)
5	22 (3400)	11 (700)	42 (4800)
6	14 (3600)	9 (850)	26 (5300)

Figures in brackets represent the total number of nuclei counted.

that they are devoid of myofilaments, although typical gap junctions are apparent. Moreover, raphe cells can be distinguished from pericardial cells in that they lack labyrinth junctions. Finally, two other characteristics of this region are a higher number of [^{3}H]Tdr-labeled cells, 1.4% ± 0.2% of total number of nuclei (see Table I), and the frequent occurrence of prophasic nuclei (Fig. 4).

Myocardium

The myocardium consists of myoepithelial cells. In the center of the myocardial layer is a group of about 10–12 nonmuscle cells per section, a so-called undifferentiated line. Apart from this line, there are no cells without myofilaments in the myocardium, and the entire wall looks like a homogeneous tissue that contains only differentiated myocytes.

Myocardial cells are flattened and partly overlap one another. Cell polarity is clearly apparent; the apical and lateral sides are well characterized. Basal lamina, continuous with that on the outer face of the pericardial cells, covers the extraluminal surface of the myocytes. Many short cytoplasmic extensions face the pericardial cavity. Nuclei with rounded profiles, large nucleoli, and dispersed chromatin are located to the apical side. Both mononucleated and binucleated myocytes (Fig. 5) were observed. The diameter of the nuclei of binucleated cells is about 4 mm and the same as that of mononucleated cells. Myofibrils are located near the luminal surface. Each cell contains only one myofiber. The sarcomere banding pattern includes Z-, A-, I-, M-, and H-bands. Myofibers in neighboring cells often run in parallel, their Z-bands being in register (Fig. 6), and they never form end-to-end attachments, that is, intercalated disks. One end of each myofiber terminates at the sarcolemma of the basal surface without producing any specialized structures, and the other end lies free in the sarcoplasm. The lateral side of each myofiber is anchored in the sarcolemma at the level of the Z-band. Randomly distributed myofilaments are seen at the apical surface. Intercellular contacts include apical junctional complexes and many gap junctions scattered along the entire length of the lateral borders. Mitochondria with well-developed cristae and distinctive intramitochondrial granules are mostly located adjacent to the myofiber and throughout the central portion of each cell. Free ribosomes and glycogen particles are numerous. The Golgi apparatus, consisting of flat cisternae with peripheral sacs and surrounding vesicles, is restricted to the apical part of the cell. No T-tubules have been found, and the sarcoplasmic reticulum (SR) is poorly developed. Most of the short tubules of the SR and vesicles are closely apposed to the myofibers; some are near the lateral cell walls where they often form peripheral couplings. Autophagosomes sometimes achieve considerable dimensions and are full of lamellated and other residues. Lipid drops are seen in the sarcoplasm. Cilia are occasionally observed projecting into the lumen of the heart. From transverse sections it is clear that the cilia lack the central filaments and, therefore, they have the "9 + 0" composition. Biciliated cells (Fig. 7) are numerous. Near and generally perpendicular to the cilium-supporting basal body, a second centriole was usually observed. Centrioles and basal bodies of cilia are always found in close proximity to the Golgi apparatus (Fig. 7).

Light-microscopic autoradiography revealed that, 4 h after injection of [^{3}H]Tdr, 0 to 2 nuclei per section are labeled in the myocardium. The number of DNA-synthesizing nuclei is equal to 0.5% ± 0.1% of total number

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Figure 2. Electron micrograph of pericardial cells of *Ascidia.* The nucleus (n) is in the center of the cell, which is filled with expanded channels of rough endoplasmic reticulum (er). The centriole and cilial basal (c) are close the Golgi complex (G) at the apical surface. The cells are joined by apical junctional complexes (ac) and labyrinth junctions (lj). The basal surface is covered with basal lamina (arrows). a, autophagosome. Scale bar = 2 mm.

Figure 3. Electron micrograph showing a labyrinth junction between two pericardial cells. Scale bar = 1 mm.

Figure 4. Electron-microscopic autoradiograph of the raphe region of the *Ascidia* heart fixed 4 h after injection of [^{3}H]Tdr. The photograph represents a rare case with two labeled (arrows) and two prophasic (pn) nuclei in one section. Arrowheads mark gap junctions; note the lack of labyrinth junctions and contractile fibers. Scale bar = 2 mm.

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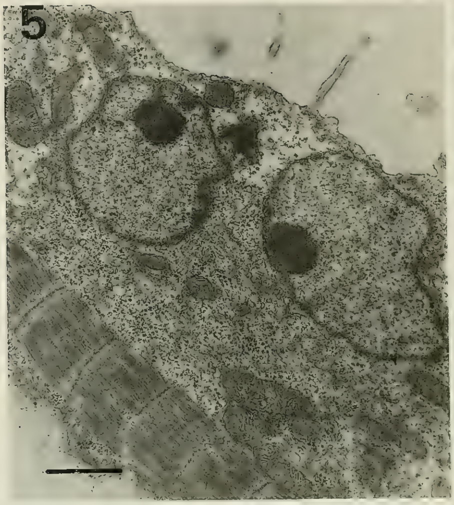

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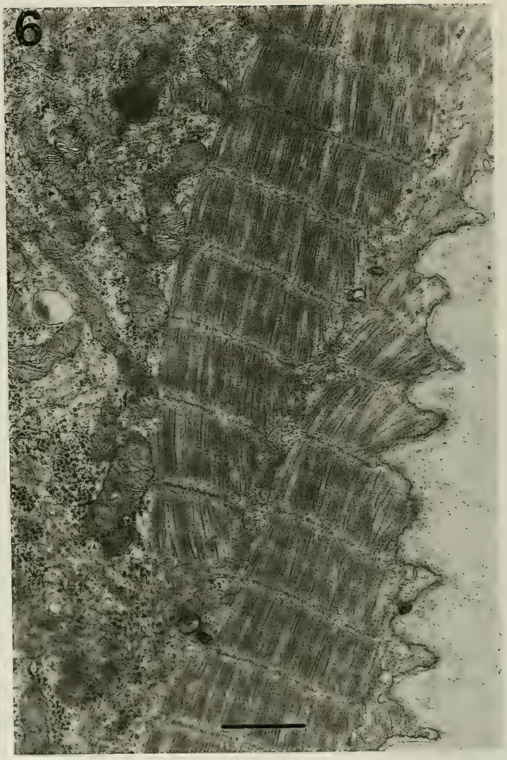

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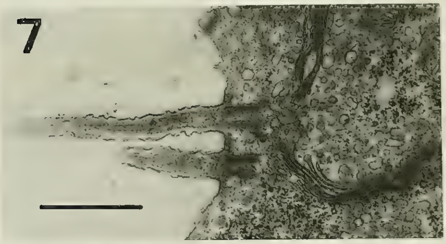

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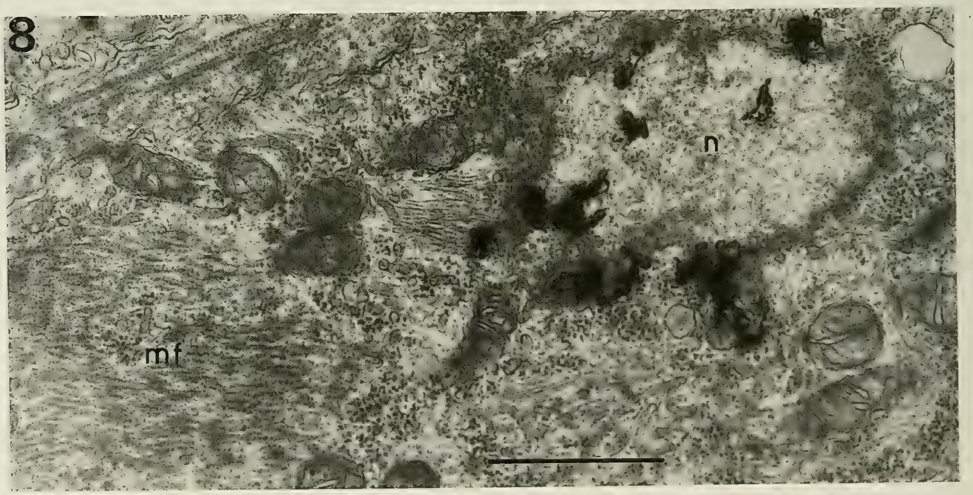

of nuclei (see Table I). These rare, labeled nuclei are apparently randomly distributed along both myocardial layers. Electron micrographs of radioautographs demonstrate that [^{3}H]Tdr-labeled nuclei in the myocardium are the nuclei of myofibril-containing cells (Fig. 8). These labeled cells have no morphological differences from other cardiomyocytes. No myonuclei undergoing mitosis were found.

In the undifferentiated line (see above), the cells contain a number of standard organelles. The intercellular contacts are represented by gap junctions in addition to apical junctional complexes. Evidence of incorporation of [^{3}H]Tdr was not observed over these cell nuclei. There is no indication that these cells remain undifferentiated to constitute a population of progenitor cells.

Discussion

In general, the ultrastructure of the heart cells of *Ascidia obliqua* is identical to that described for other ascidians (Kriebel, 1968; Kalk, 1970; Jones, 1971; Lorber and Rayns, 1972; Oliphant and Cloney, 1972; Weiss *et al.,* 1976; Nunzi *et al.,* 1979; Kriebel and Hanna, 1980). In all the cited studies, attention was focused on the structures responsible for the contractile activity of the myocytes. In the present study we examined the ascidian heart in terms of its generative abilities and its relation to the vertebrate heart.

The myoepithelial nature of the ascidian myocardium, with its clearly expressed pattern of polarity, is to some extent reminiscent of that of the vertebrate embryonic heart (Hirakow, 1985; Peng *et al.,* 1990). Vertebrate cardiac myoblasts are developed from the splanchnic mesoderm without loss of their epithelial organization, and early cardiomyocytes are, in fact, myoepithelial cells (Manasek, 1973).

A remarkable feature shared by the myocardial cells of ascidians and vertebrates is the presence of a developed secretory apparatus. It is well established in vertebrates that—unlike the skeletal muscles, which probably pass through a secretory phase at some very early stages of development (see Manasek, 1979, for discussion)—the cardiomyocytes are bifunctional, combining contractile and endocrine activity at all ages. Two phases can be distinguished in their secretory activity. The first is responsible for the production of cardiac jelly by embryonic cardiac muscle (Nakamura and Manasek, 1978). In the second phase, membrane-limited, electron-dense granules containing the so-called atrial natriuretic factor appear in mature cardiomyocytes (Aardal and Helle, 1991). We did not find such granules in the heart cells of *Ascidia obliqua,* nor have they been reported in ascidian hearts by other authors. However, the pericardial cells of ascidians contain expanded channels of rough endoplasmic reticulum and well-developed Golgi complexes; myocardial cells retain the latter, which provides evidence that, in ascidians, both pericardial and myocardial cells perform secretory functions. Lack of biochemical data prevents us from discussing the nature of the substances secreted by these cells. The essential point here is that ascidian cardiomyocytes are apparently bifunctional and, in this respect, they are analogous to all vertebrate heart-muscle cells.

The most significant similarity we found between the ascidian myocardium and the vertebrate myocardium is the capacity for DNA synthesis in mature myofibril-containing myocytes. Whether this replication process is accompanied by cell division in the ascidian heart is uncertain, because we failed to observed mitotic figures. Ascidian pericardial cells are always mononucleated, whereas binucleated cells are abundant in the ascidian myocardium. Because both pericardial and myocardial cells are capable of DNA synthesis, we can assume that, in the former, the replication of DNA is followed by complete mitosis, but in the latter, only karyokinesis occurs. The presence of highly organized myofibers is an obstacle to the progression of cytokinesis, and ascidians lack the complex mechanism of myofiber disassembly with Z-bands resorbsion that is found in some vertebrate cardiomyocytes (Rumyantsev, 1991). Fusion of cells cannot be responsible for binucleation in the ascidian myocardium, because the synthesis of DNA in myocytes is continuous. Amitotic division of nuclei is also an unlikely mechanism, because it would produce some polyploid nuclei.

The presence of centrioles in ascidian cardiomyocytes may be regarded as indirect evidence of mitotic activity in these cells. In a comparative study of chick skeletal and cardiac muscles, Przybylski (1971) found that myocardial

Figures 5–8. Electron micrographs of myocardial cells of *Ascidia.* Scale bars = 1 mm.

Figure 5. A binucleated myocardial cell.

Figure 6. Myofibers in neighboring myocytes are aligned in parallel, their Z-bands being in register, and they do not form contacts of the intercalated-disk type.

Figure 7. Two cilia with their basal bodies close to the Golgi apparatus.

Figure 8. Electron-microscopic autoradiograph of a myocardial cell from an ascidian heart. Numerous silver grains can be seen over the nucleus (n), indicating the presence of radioactive DNA synthesized by this differentiated cell, which contains myofibrils (mf).

cells contain centrioles and cilia at all stages of differentiation, in contrast to skeletal myoblasts, which lose these organelles soon after fusion. Przybylski attributed these results to the inability of skeletal muscle nuclei to divide mitotically and the retention of this ability by myocardial cells.

Thus the ascidian myocardium is apparently similar to the early vertebrate myocardium in its myoepithelial organization; its possession of well-developed secretory apparatus, centrioles, and cilia; and, most importantly, its capacity for the synthesis of DNA and probably for nuclear division.

According to our estimates, the raphe region comprises about 3 times more [^{3}H]Tdr-labeled nuclei than are found in the myocardium or the pericardium. The frequent occurrence of DNA-synthesizing and prophasic nuclei in the raphe region points to the existence of a zone of active proliferation of cells and, very probably, a source for replenishment of myocytes. Some morphological features, such as the loss of labyrinth junctions and the acquisition of gap junctions, provide evidence for the possibility that cells in the raphe region are premyoblasts. Although these cells do not contain myofilaments, Nunzi *et al.* (1979) theorize that membrane changes observed during the differentiation of ascidian cardiomyocytes precede the formation of myofibrils. We do not see now what kind of experiments could verify our assumption that cells of the raphe region have a myoblastic nature. If the assumption is true, the ascidian myocardium, unlike the vertebrate myocardium, cannot be related to the pure noncambial growing population of cells. A possible double mechanism for replenishing cardiomyocytes in the ascidian heart might combine their own reproduction with the persistent transformation of pericardial cells into myocardial cells in the raphe region.

The nonmuscle cells in the center of the ascidian myocardium, the so-called undifferentiated line, can hardly be considered to form a cambial zone: there is no evidence here of the proliferation of cells and, more significantly, not all tunicates possess such a line (Weiss *et al.,* 1976).

It is of interest to compare our results with those from electrophysiological studies that demonstrate similarities in action potentials and spread of excitations in ascidian and mammalian myocardia (Kriebel, 1967, 1970). In addition, Kriebel (1967) showed that neither the raphe region nor the undifferentiated line of the ascidian heart is required for conduction, though the undifferented line, unlike the raphe, can conduct a wave of excitation. Whereas the role of a cambial zone can be suggested for the raphe region, the function of the undifferentiated line remains altogether unclear.

The crayfish is another invertebrate for which data are available on the cell mechanisms of heart-muscle growth. Crayfish myocardium is nonhomologous to the myocardium of the deuterostomes. Crayfish heart muscle is analogous to vertebrate skeletal muscle in that it contains undifferentiated mononucleated satellite cells capable of DNA synthesis and fusion with the multinucleated myofiber (Martynova, 1993). Taking into account our preliminary (unpublished) data that myogenic stem cells are also present in the heart muscle of molluscs and brachiopods and that their differentiated cardiomyocytes lack the capability for DNA synthesis, we can assume that a true cardiac muscle has been developed only once in evolution—in deuterostomes.

Acknowledgments

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Univacuolar Refractile Hemocytes From the Tunicate *Ciona intestinalis* Are Cytotoxic for Mammalian Erythrocytes *in vitro*

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Abstract. A discontinuous, Percoll density gradient was used to separate hemocyte populations from the hemolymph of *Ciona intestinalis.* Hemocytes from each band were examined for their frequency, morphology, and cytotoxic activity against rabbit and sheep erythrocytes; results were expressed as a percentage of hemolysis. Statistical analysis revealed that only the "univacuolar" granulocytes from Band 5, which contain a vacuole of refractile material, were cytotoxic. Cytotoxic activity was inhibited by sphingomyelin. For the first time in tunicates, lytic activity against erythrocytes was assessed by an assay based on plaque-forming cells. Plaques of lysis were revealed against rabbit erythrocytes but not against sheep erythrocytes.

Introduction

Natural cytotoxicity against allogeneic and xenogeneic cells including mammalian tumor cell lines has been demonstrated by *in vitro* assays using hemocytes from several invertebrate groups. The cell-killing mechanism can involve soluble cytolysins or require contact between effector and target cells (reviewed in Canicattì, 1990; Parrinello and Arizza, 1992; Lotzovà, 1993). Cytotoxic cells from annelids, echinoderms, and molluscs have been compared to natural killer cells in vertebrates (Tyson and Jenkin, 1974; Decker *et al.,* 1981; Luquet and Le Clerc, 1983; Söderhäll *et al.,* 1985; Franceschi *et al.,* 1991; Porchet-Henneré *et al.,* 1992; Suzuki and Cooper, 1995). In tunicates, hemocytes from solitary ascidians display cytotoxic activity *in vitro* when examined in xenogeneic or allogeneic combinations (Fuke, 1980; Kelly *et al.,* 1992). Mammalian erythrocytes (Parrinello and Arizza, 1992; Parrinello *et al.,* 1993, 1995) and tumor cell lines (Peddie and Smith, 1993, 1994) were used as targets to reveal *in vitro* cytotoxic activity by hemocytes from the solitary ascidian *Ciona intestinalis.* Cytotoxicity of a total hemocyte population, assayed against sheep erythrocytes (SE), is a calcium-dependent, cell-mediated reaction that occurs rapidly (15–30 min) at 25°-37°C, is maximal at a 1:3 effector/target cell ratio, and is inhibited by sphingomyelin (Parrinello *et al.,* 1993, 1995). The hemocyte cytotoxic activity (HCA) against mouse tumor cell lines is cell-mediated, Ca^{++}-dependent, and occurred within 15 min at 20°C, at an effector/target cell ratio of 10:1 when an enriched hemocyte population was used. HCA involves energy metabolism, requires an intact cytoskeleton, and depends upon active secretory processes in the effector cells (Peddie and Smith, 1993, 1994). Reported differences in requirements for effector/target cell ratios suggest that anti-erythrocyte and anti-tumor HCAs possess distinct characteristics with respect to mechanisms.

The hemolymph of the sea squirt contains several hemocyte types: stem cells, nonvacuolar hyaline amoebocytes, vacuolar and granular amoebocytes, and vacuolated (signet-ring, morula, compartment, orange) cells (Wright, 1980; Rowley, 1981, 1982; Rowley *et al.,* 1984). Hemocyte populations, separated by density gradient centrifugation, were compared for their cytotoxic activity against tumors; an *in vitro* eterochromasia assay (Peddie and Smith, 1993) was used to assess cytotoxicity. Only cell bands that contained granular phagocytic and hyaline nonphagocytic amoebocytes exhibited cytotoxicity against tumor cells. On the other hand, preliminary experiments upon biological activities of separated hemocytes (Parrinello *et al.,* 1992, 1995) indicated that morula cells, uni-

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vacuolar cells, or both—contained in the same band—were cytotoxic against erythrocytes and induced measurable hemoglobin release. In this paper we describe a separation method that we have developed and examine the hemocyte type responsible for anti-erythrocyte HCA. In addition, for the first time in tunicates, we use an assay of plaque-forming cells to reveal the cytotoxic cell. Finally, another variable to determine whether the cytotoxic cell is inhibited by sphingomyelin has been included.

Materials and Methods

Tunicates, collection of hemolymph, preparation of hemocyte suspensions, and cell viability

Specimens of *Ciona intestinalis* were collected from the Gulf of Palermo, maintained at 20°C in a closed-circuit marine aquarium, and fed with a marine invertebrate diet (Liquifry, Interpet Ltd., England) until ready for experiments. Hemolymph was withdrawn from the heart with a 1-ml syringe into an equal volume of anticoagulant solution (artificial seawater without $CaCl_2$ and $MgCl_2$ but enriched with 10 m*M* EDTA, pH 7.0: FSW-EDTA). Hemocyte suspensions were prepared as described by Peddie and Smith (1993, 1994). Hemocytes from individual tunicates, after centrifugation at 800 × *g* for 10 min, were washed (twice) with FSW-EDTA and finally suspended in marine solution (MS). This solution (12 m*M* $CaCl_2 \cdot 6H_2O$; 11 m*M* KCl; 26 m*M* $MgCl_2 \cdot 6H_2O$; 43 m*M* Tris; 38 m*M* HCl; 0.4 *M* NaCl, pH 8) is isosmotic with hemocytes (1090 mOsm kg^{-1}). Cells were counted using an improved Neubauer hemocytometer. Hemocyte suspensions containing 15×10^6 cells/ml were prepared. Hemocyte mortality, evaluated by the eosin-y dye (0.5% in MS) exclusion test, was less than 4.0% in each experimental procedure.

Preparation of discontinuous density gradient

We used a Percoll density gradient described by Cammarata *et al.* (1993) with slight modifications. In brief, Percoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) was dialyzed against FSW-EDTA. Equilibrated Percoll solution was adjusted with FSW-EDTA to a final concentration of 90% (density, 1.122 g/ml). This stock solution was mixed with an appropriate volume of FSW-EDTA to prepare solutions of decreasing densities (1.105, 1.098, 1.090, 1.079, 1.071, and 1.060 g/ml). Aliquots of 1 ml of each solution were overlaid sequentially into 10-ml centrifuge tubes. Then, 4 ml of hemocyte suspension ($5.0–10.0 \times 10^6$ per ml) was diluted v/v with FSW-EDTA and layered onto the gradient, and the tube was centrifuged in a swing-out rotor at 800 × *g* for 15 min at 7°C. Bands of cells were collected by gentle aspiration from the top of the tube and washed three times in sterile FSW-EDTA before being suspended in MS. After observing the HCA of each distinct band of cells, we shortened the separation method by using a 55% Percoll density gradient that specifically enriches the cytotoxic population: whole hemocyte suspensions were layered onto the Percoll solution, the tubes were centrifuged in a swing-out rotor at 800 × *g* for 30 min at 7°C, and the cell band on the bottom of the gradient was gently collected.

Preparation of erythrocyte targets

Rabbit erythrocytes (RE) were purchased from Sclavo (Siena, Italy), and sheep erythrocytes (SE) were provided by the Zooprophylaxis Institute (Palermo, Italy). Erythrocytes were washed three times in phosphate-buffered saline (PBS) and, after centrifugation for 10 min at 400 × *g*, resuspended in 10 ml MS at the required concentration. Despite the high osmolality of the medium, significant cell lysis was not found when erythrocytes were suspended in MS.

Hemocyte cytotoxic assay (HCA)

The cytotoxic assay has been previously described (Parrinello *et al.*, 1993, 1995). In brief, 200 μl of hemocyte suspensions ($2–3 \times 10^6$ unfractionated cells or $0.5–1 \times 10^6$ fractionated cells) in MS was mixed with an equal volume of freshly prepared SE or RE suspension (8×10^6 cells) in MS. Hemocyte counts were determined in the final volume of the reaction mixture. The mixture was incubated with continuous and moderate shaking at 25°C for 1 h. At the end of the incubation, the supernatant was separated and the amount of the released hemoglobin (Hb) was estimated by reading the absorbance at 541 nm in a spectrophotometer. The degree of hemolysis was determined according to the equation:

$$\text{Percent hemolysis} = \frac{\text{measured release} - \text{spontaneous release}}{\text{complete release} - \text{spontaneous release}} \times 100$$

Complete hemolysis was obtained by preparing an erythrocyte suspension in distilled water. Appropriate controls showed that hemocyte mortality in MS medium, under experimental conditions, was low (0%-5%). Control erythrocyte suspensions were also prepared in the same medium and incubated as reaction mixtures: spontaneous hemoglobin release never exceeded 5% of the total release. For each experiment three samples were prepared, producing values representing a mean ± SD.

Assays for inhibition of cytotoxicity

Sphingomyelin (from bovine brain), phosphatidylcholine, phosphatidylserine, cholesterol, phosphorylcholine, and phosphorylethanolamine (Sigma) were dissolved in MS. Phosphatidylcholine, phosphatidylserine, sphingomyelin (SM), and cholesterol were relatively insoluble. For these compounds, stock solutions were briefly sonicated (Vibra-Cell, Sonics & Materials Inc., Danbury, CT). Hemocyte suspensions were prepared in the medium containing compounds at various concentrations. After 20 min preincubation, erythrocyte suspensions (prepared in MS containing the same amount of inhibitor) were added at various effector/target cell (E/T) ratios for cytotoxic assays. Lysis due to chemicals alone was measured as controls.

Plaque-forming-cell (PFC) assay

We have developed a PFC assay using tunicate hemocyte effectors. The technique required only slight modifications to the procedure originally described by Cunningham and Szenberg (1968). A 50-μl sample of hemocyte suspension in MS (1×10^6 ml) was mixed with a 50-μl suspension of erythrocytes (SE and RE) in MS (5%). The reaction mixture was gently layered in a Cunningham-Szenberg chamber formed by a thin strip of adhesive tape placed between the borders of a coverslip and a microscope slide. After 15 min of incubation at 20°C, the cell mixture was examined under a phase-contrast microscope.

As part of the PFC assay, inhibition experiments were performed by adding 25 μg/ml SM (final concentration) to the medium of the HCA assay, just before filling up the chamber. As controls, (1) the volume of SM stock solution added to the reaction mixture was replaced with a same volume of MS, and (2) the same amount of SM was contained in both the medium used for effector cells and the erythrocyte suspension that was examined separately.

Statistical analysis

To express the functional relationships between hemolysis degree (%) and cell types (cell number) enriched by density gradient, the logistic regression model was used (Hosmer and Lemeshow, 1989). This model is helpful when the dependent variable varies within a particular lower and upper limit. The functional relationships (Hb absorbance values/cell number) were also examined by means of a multiple linear regression equation (Sokal and Rohlf, 1981). Statistical significance was determined by reference to the *t*-distribution, using the standard error of the correlation coefficient.

Results

Fractionation on a Percoll discontinuous density gradient of Ciona *hemolymph: observations on separated hemocytes*

The total population of hemocytes was partitioned into six distinct, discrete bands (B1–B6). Cell bands included hemocyte types that were identified, by morphology, according to Rowley (1981, 1982; Rowley *et al.,* 1984) and De Leo (1992), with slight modifications in terminology. The following well-established cell types were recognized: stem cell (Fig. 1A); pigment cell (Fig. 1B); hyaline amoebocyte (Fig. 1C); granular amoebocyte (Fig. 1D); univacuolar refractile granulocyte (URG), with a single prominent refractile inclusion in a large vacuole that nearly filled the whole cell (Fig. 1E); signet-ring cell, similar to a URG with nonrefractile material in the vacuole (Fig. 1F); morula cell (Fig. 1G, H). Because morula cells with large refractile granules varying in their size and number can be partitioned into distinct bands, we distinguished a type A with more than three granules (Fig. 1G), and a type B with two to three granules (Fig. 1H).

The mean number of cells in each band was calculated from five experiments, after fractionation of $20–40 \times 10^6$ hemocytes. Although each band was mainly enriched for certain hemocyte types, homogeneous populations were not separated. In Table I, the percentages of each hemocyte type, as evaluated by examining washed hemocytes before density-gradient separation, are reported. B1 consisted mostly of hyaline amoebocytes (76%), but stem cells and signet ring cells were present; B2 was richer in hyaline amoebocytes (57%), but stem cells and granular amoebocytes were also included; B3 consisted primarily of granular amoebocytes (69%) but hyaline amoebocytes

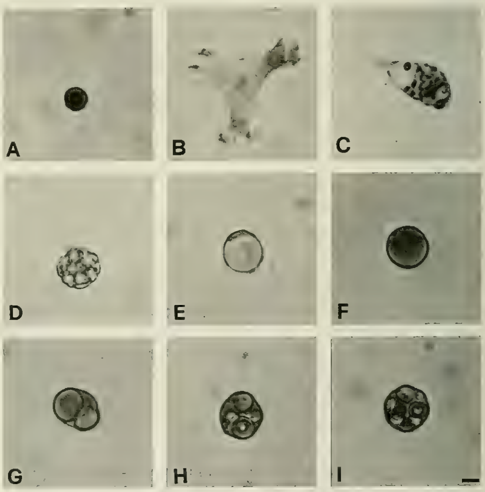

Figure 1. *Ciona intestinalis* hemocytes from a Percoll density gradient. Phase-contrast microscopy, scale bars 4 μm. (A) Stem cell; (B) hyaline amoebocyte; (C) granular amoebocyte; (D) pigment cell; (E) signet-ring cell; (F) univacuolar refractile granulocyte (URG); (G) early B-type morula cell; (H) B-type morula cell; (I) A-type morula cell.

Table I

Differential distribution of cytotoxic hemocytes from Ciona intestinalis *in bands obtained from a discontinuous Percoll density gradient*

Hemocyte band	Hemocyte type (% ± SD; $n = 5$)									HCA[2] (%)
	Cell count ($\times 10^6$)	Stem cells	Hyaline amoebocyte	Signet ring cell	Granular amoebocytes	Morula cells[1]		Univacuolar refractile granulocytes	Pigment cells	
						A-type	B-type			
B1	5.7	13 ± 5.0	76 ± 7.0	11 ± 10.0	—	—	—	—	—	—
B2	6.0	14 ± 6.0	57 ± 12.0	9 ± 3.0	20 ± 4.0	—	—	—	—	—
B3	3.3	1 ± 0.5	22 ± 0.5	8 ± 7.0	69 ± 10.0	—	—	—	—	—
B4	5.1	—	—	—	48 ± 3.0	40 ± 8.0	12 ± 4.0	—	—	—
B5	8.1	—	—	—	—	8 ± 3.0	52 ± 7.0	40 ± 12.0	—	88
B6	1.5	—	—	—	—	5 ± 1.0	80 ± 18.0	10 ± 2.0	5 ± 1.0	12
Unfractionated		5 ± 2.0	18 ± 3.0	16 ± 4.0	20 ± 2.7	12 ± 3.0	19 ± 5.0	10 ± 5.0	<1	47

[1] A-type contains more than 3 granules; B-type contains 2–3 granules.

[2] HCA = Hemocyte cytotoxic activity (% of hemolysis) of 3×10^6/ml hemocytes from each band assayed against rabbit erythrocytes.

(22%) were also present; B4 was composed mainly of granular amoebocytes (48%) and A-type morula cells 40%; B5 contained B-type morula cells (52%) and URGs (40%); finally, B6 was largely (80%) made up of B-type morula cells.

The cell band obtained in a monostep hemocyte separation on 55% Percoll discontinuous density gradient contained univacuolar refractile granulocytes (43%) and B-type morula cells (57%).

Cytotoxic activity of fractionated hemocytes against rabbit erythrocytes

Comparison of HCA by each separated cell band (Table I) showed that cytotoxic cells were enriched in band B5 containing (40%) URGs and B-type morula cells (52%). Statistical analysis by a paired t test revealed that the percentage of hemolysis by the B5 cell population was significantly higher than that observed with the B6 cell population, formed primarily (80%) of B-type morula cells ($P < 0.01$). The other bands enriched in B-type morula cells (B4), granular amoebocytes (B3), and hyaline amoebocytes (B1) exhibited no significant cytotoxic activity ($P > 0.005$; Table I). Nonphagocytic hyaline amoebocytes (B1, B2) and phagocytic amoebocytes (B2, B3), although present in sufficient numbers to achieve an optimal E:T ratio, failed to show anti-RE cytotoxic activity. Stem cells were not present in sufficient numbers to reach an equivalent E:T ratio. The hemocyte types obtained by density-gradient separation are shown in Figure 1.

To describe whether the variability in cytotoxic activity is an effect of UGRs or B-type morula cells contained in B5, a logistic regression model was superimposed on the data. A close functional relationship (goodness of fit = 0.88) is evident between degree of hemolysis and URG number in enriched populations (Band 5) from 16 different experiments (Fig. 2a), but no relationship (goodness of fit = 0.058) is seen with morula cells (B-type) contained in that band (Fig. 2b). In addition, when the relationships between cell numbers and absorbance values of released hemoglobin were examined by means of a linear multiple regression equation, the close relationship between hemolysis and URGs ($r = 0.885$) was supported ($P < 0.001$), but no correlation was found for the morula cells ($P > 0.05$). As a result, all further experiments were performed using hemocyte populations enriched in URGs by monostep separation.

Hemocyte cytotoxic activity at various effector/target ratios

To examine the cytotoxic mechanism, the HCA was assayed at different E/T ratios. The curves, obtained by plotting the numbers of unfractionated hemocytes against the hemolysis of RE, are sigmoidal (Fig. 3). A significant rise in hemolysis was found when 0.9×10^6/ml hemocytes reacted against 22.5×10^6/ml target cells (E/T ratio 1:24); the highest value (about 80%) was reached using 7.5–3.8×10^6 hemocytes/ml (E/T ratio 1:3, 1:6). The results were different in that a hyperbola was formed by the curve obtained by plotting enriched hemocyte populations from B5 *vs.* hemolysis: a significant rise in hemolysis was found when 22×10^5 cells/ml were mixed with 22.5×10^6/ml RE (E/T ratio 1:96).

Inhibition of lysis by sphingomyelin

With the aim of examining the cytolytic mechanism of an enriched population of effector hemocytes, the interaction between erythrocyte membrane lipids and lysins was examined by inhibition experiments.

In *Ciona intestinalis,* bovine erythrocyte sphingomyelin inhibits the cytotoxic activity of unfractionated hemocytes

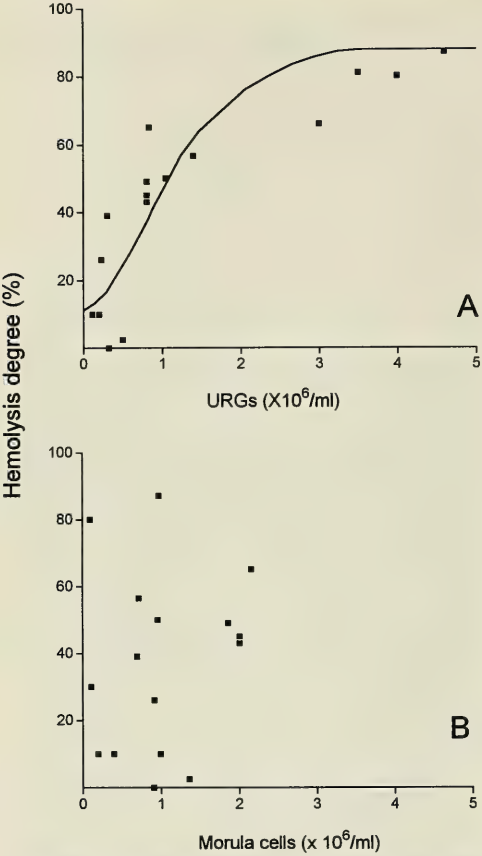

Figure 2. Anti-rabbit erythrocyte cytotoxic activity of *Ciona intestinalis* hemocyte types enriched into Band 5 of a Percoll density gradient. The curves represent the logistic regression model to describe the relationship between degree of hemolysis and different numbers of (A) URGs (R = 0.88) or (B) B-type morula cells (R = 0.058).

as well as of URGs contained in band 5 (Fig. 2). Inhibition was evident at 25 μg/ml SM and at various E/T ratios, and is apparently not due to toxic effects on effector cells because the percentage of dead cells was less than 5% in hemocyte suspensions containing sphingomyelin at the inhibiting concentration. The inhibitory capacity is evident at an E/T ratio of 1:12, which is optimal for hemolysis, when compared to the profile of noninhibited reaction mixtures. The degree of inhibition ranged from about 80% (when using a 0.25 μg/ml inhibitor) to about 95% (for a 250 μg/ml inhibitor). To act as an inhibitor, SM must be present throughout the reaction, whereas the separate pretreatment of effector or target cells was ineffective (data not shown). Phosphatidylcholine, phosphatidylserine, phosphatydil-ethanolamine, and cholesterol were inactive even when assayed at their highest concentrations (data not shown).

Hemocyte cytotoxic activity assayed against sheep erythrocytes

The URGs from B5 lysed SE to a lesser extent than they lysed RE (Fig. 2). However, the slope of the curves

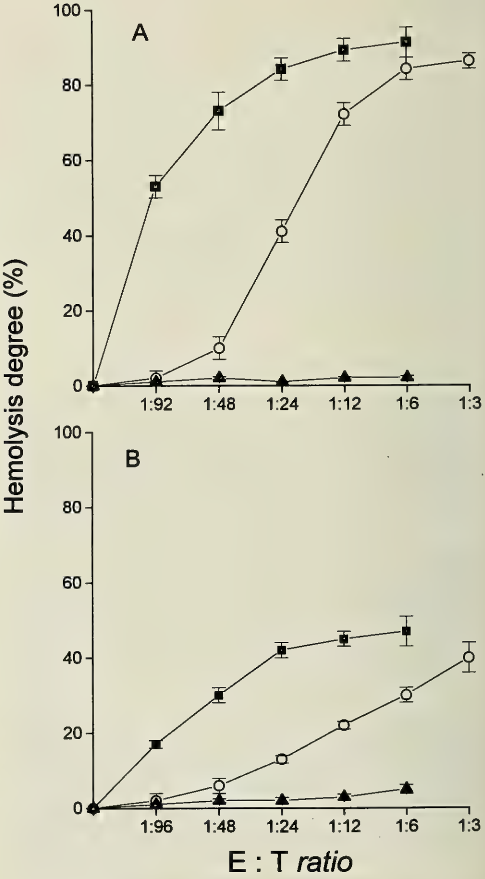

Figure 3. Lytic activity of *Ciona intestinalis* unfractionated O—O and fractionated (B5) ■—■ hemocytes assayed against rabbit (A) or sheep (B) erythrocytes at various effector/target ratios. Inhibitory effect of sphingomyelin (25 μg/ml) ▲—▲.

obtained at different ratios of effector/target cells (RE and SE) are similar, while sphyngomielin was also an inhibitor of anti-SE HCA.

Lysis as revealed by plaque formation

To confirm that URGs from B5 were responsible for cytotoxic activity, a plaque-forming assay was performed. For the first time, ascidian hemocytes that form plaques of lysis are shown (Fig. 4). Plaques were observed in the B5 hemocyte/RE mixture layered in a Cunningham-Szenberg chamber (Fig. 4A), whereas no lysis was observed in controls of erythrocytes alone (Fig. 4B). In three experiments, plaques constituted 10%-20% of the effector cells. On the contrary, plaques were not found when SE were used as a target. At high magnification, URGs are seen to be located in the center of plaques (Fig. 4C and inset). Addition of sphyngomielin at 25 μg/ml inhibited plaque formation, whereas there was no effect on hemocyte or erythrocyte suspensions used as controls.

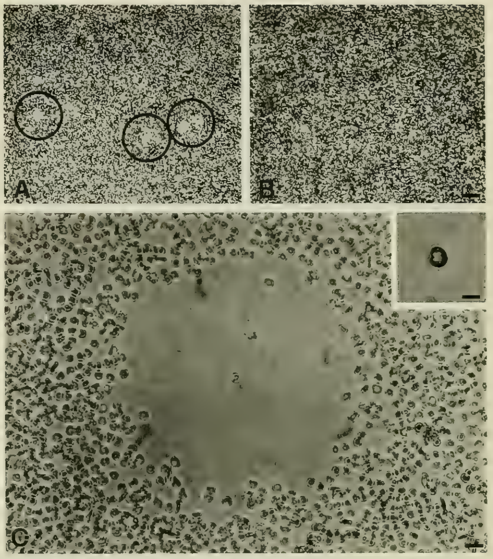

Figure 4. Plaque assay of fractionated (B5) *Ciona intestinalis* hemocytes capable of secreting lytic factors against rabbit erythrocytes. (A) Plaques of lysis, bar = 560 μm; (B) monolayer of rabbit erythrocytes, bar = 560 μm; (C) plaque-forming hemocyte, bar = 40 μm; inset: cytotoxic hemocyte (univacuolar refractile granulocyte), bar = 10 μm.

Discussion

Cell-separation techniques provide a useful approach to the study of ascidian hemocyte functions (Cammarata *et al.*, 1993; Smith and Peddie, 1992). Separation of *Ciona intestinalis* hemocytes by density gradient centrifugation yielded six distinct bands of cells enriched with types formerly described by Smith and Peddie (1992) in fractionated hemolymph of this ascidian. Our light microscopy observations of the cells separated by a Percoll discontinuous density gradient supported the view put forth by De Leo (1992) that hemocytes characterized by a large vacuole, which contains refractile inclusion surrounded by a thin rim of cytoplasm, can be regarded as "vacuolar granulocytes." We called this hemocyte type a "univacuolar refractile granulocyte" (URGs) to distinguish it from another univacuolar hemocyte, called a "signet-ring cell," that did not contain refractile inclusions. Differences in the density of the vacuolar inclusion may be responsible for the distribution of these cell types in distinct bands: signet-ring cells in B1 (11% of total hemocytes) and URGs in B5 (40% of total hemocytes). Only URGs from band 5 appeared to be cytotoxic for erythrocyte targets.

The hemocyte type we defined as a univacuolar refractile granulocyte on the basis of its vacuolar content was previously described by Rowley (1981) as a "compartment cell." However, the latter term leads to confusion in hemocyte classification because it has also been used to indicate a multivacuolated hemocyte of other ascidian species (see De Leo, 1992).

In addition, our so-called "morula cells" contained various amounts of globular materials causing them to accumulate in different gradient density bands. The difference was due only to the size and number of their granules, so we referred to them collectively as morula cells. We did, however, distinguished them as either "A-type" or "B-type," depending upon the number of granules. The existence of morula cells in multiple fractions suggests that these cells may have multiple states. Rowley *et al.* (1984) suggested that there may be interrelationships between the "multivacuolated compartment cell" (equivalent to the B-type morula cell) and the more vacuolated morula cell (equivalent to the A-type). Our light microscopy observations of the separated hemocytes (Fig. 1, G–I) support this hypothesis.

The anti-erythrocyte cytotoxic activity examined in this paper and the anti-tumor cytotoxicity reported by Peddie and Smith (1993) reside in different cell bands. Comparison with cytotoxic activity by hemocytes from other bands, as well as the statistical estimation of a logistic regression model, demonstrates that the URGs from band 5 lysed erythrocytes; in contrast Smith and Peddie (1992) found that only cell bands containing granular phagocytes and hyaline nonphagocytic amoebocytes killed tumor cells. In our experiments anti-erythrocyte activity was never found in bands B1, B2, and B3 enriched for hyaline or granular amoebocytes. The existence of morula cells in multiple fractions suggests that there may be multiple states of these cells, so the active fraction might have contained unique (or activated) morula cells that were responsible for cytotoxicity. However, the B-type morula cells, numerous in the active band 5, were not considered cytotoxic against RE because the logistic regression model, used to describe the effects of different numbers of these cells, showed that hemolytic activity is related only to increased numbers of URGs. In addition, B6, which was mainly (80%) enriched in B-type morula cells, was not cytotoxic.

On the basis of cytotoxic activity, no functional relationship can be identified between the two forms of univacuolar hemocytes. However, a morphological relationship between refractile (URGs) and nonrefractile (signet-ring cells) univacuolar hemocytes involved in inflammatory reactions of the tunic (De Leo *et al.,* 1992) is suggested by light and electron microscopy observations: the refractile material of URGs undergoes changes in electron density, becoming electron-transparent like the material in the vacuole of signet-ring cells.

If increasing numbers of unfractionated hemocytes are plotted against hemolysis of RE, the resulting curve is sigmoidal (Parinello *et al.,* 1995). When various effector/target cell ratios were plotted against the degree of hemolysis caused by hemocytes unfractionated or fractionated in B5, it is evident that enriched populations of URGs were more efficient in producing hemolysis. In fact, the degree of hemolysis was greater than 50% when a 1:96 effector/target cell ratio was reached, whereas unfractionated cells caused 40% hemolysis at higher (1:24) E/T ratios. The enrichment in effector cells probably allowed more frequent contacts with targets, and more cytolysins could be released.

The effector cells were not specific for RE, and they lysed sheep erythrocytes. However, hemolytic activity was significantly ($P < 0.01$) lower against SE than against RE. According to a previous paper (Parrinello *et al.,* 1995) on unfractionated hemocytes, sphingomyelin strongly inhibited cytotoxic activity of populations enriched in effector cells when assayed against both RE and SE. Although sphingomyelin was an inhibitor of SE lysis, the differences reported to date in the lytic activity of effector cells indicate that the composition of the target cell membrane affects the lytic mechanisms.

By using an assay based on plaque formation we confirmed that URGs are the hemocyte type cytotoxic for rabbit erythrocyte targets. For the first time in tunicates, we show hemocytes that form plaques. The erythrolytic cell, at the center of the plaque, is characterized by a vacuole that contains refractile material and occupies almost all the cytoplasm. In addition, plaque formation suggests that cytotoxic cells may be activated by contact with targets and release molecules that lyse neighboring erythrocytes. Because the percentage of plaques observed against RE was low (20% of the effector hemocytes in the reaction mixture), the ability to release cytolysins probably depends on differences in stages of hemocyte differentiation, on activating mechanisms in the cell membrane, or on both factors.

The finding that plaques are not formed against SE appears to agree with a previous (Parrinello *et al.,* 1993) in which we were not able to show hemocyte-free anti-SE lysins, and supports the view that differences in the composition of the erythrocyte membrane affect the lysis.

Apparently our results differ from those reported by Peddie and Smith (1993) in that effector cells against tumor target cells are contained in an enriched population composed of nonphagocytic and phagocytic amoebocytes. *In vitro* cytotoxic activity is probably a property of diverse hemocyte types. This activity could become evident in connection with the membrane composition of target cells, and it could also depend upon secondary products of hemocyte activities—*i.e.,* phagocytosis. Finally, the role of the method used to reveal the cytotoxic activity cannot be excluded. Further research is needed and is now in progress to isolate and characterize the molecule or molecules responsible for cytolysis.

Acknowledgments

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